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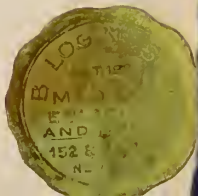
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THE  
JOURNAL OF INFECTIOUS DISEASES





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LUDVIG HEKTOEN AND EDWIN O. JORDAN

IN CONJUNCTION WITH

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W. T. SEDGWICK

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# *The* Journal of Infectious Diseases

FOUNDED BY THE MEMORIAL INSTITUTE FOR INFECTIOUS DISEASES

VOL. 6

February 18, 1909

No. 1

## THE EPITHELIAL CELL CHANGES IN MEASLES.\*

JAMES EWING.

(From Cornell University Medical College, New York City.)

NEUMANN<sup>1</sup> in 1880, writing on the subject of the histology of measles, expressed his surprise that so little attention had been paid to this interesting subject. The only definite observation which he was able to find was that of Cornil and Ranvier<sup>2</sup> who described in the skin engorgement of the vessels and the presence of some wandering cells and pigment granules in the papillae and Malpighian layer. Contrary to Cornil, Neumann did not detect any leucocytes in the epithelium, but found the cellular infiltration limited to the subepithelial tissue and localized in a rather specific manner about the hair follicles and sebaceous and sweat glands. Likewise Mayr,<sup>3</sup> as well as Hebra,<sup>4</sup> located the morbid process chiefly in the sebaceous glands, finding the hair follicles only occasionally and accidentally involved. Kaposi<sup>5</sup> dismissed the subject with the statement that the microscope

\* Received for publication November 15, 1908.

<sup>1</sup> *Med. Jahrb.*, 1882, p. 157.

<sup>2</sup> *Anat. Path.*, 2, p. 761; also *Path. Hist.*, London, 1886, 2, p. 651.

<sup>3</sup> *Hebra's Diseases of Skin*, London, 1866, 1, p. 177.

<sup>4</sup> *Lehrbuch d. Hautkrankheiten*, Erlangen, 1872, 2, p. 137.

<sup>5</sup> *Path. et Traité des malad. de la peau*, Paris, 1891, 1, p. 250.

*Robert*  
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had not been able to show, either in the epidermis or in the papillae, any lesion which pointed to a proliferation of cells.

The first report of a definite effort to examine in detail and with competent methods the cutaneous lesions of measles appears to be that of Catrin,<sup>1</sup> who in 1890 presented a full report of the study of a single case coming to autopsy on the fourth day at the height of a maculo-papular eruption. His general conclusion was that the localized maculo-papular lesions reduce themselves purely and simply to a lymphoid infiltration about the arterioles, the hair follicles, and the sebaceous and sweat glands. In addition to this process, which had been recognized by others, he added the description of a new lesion in the form of vesicles, "phlyctenes," in various stages of development. Some of these were superficial and consisted of a separation of the epithelial layers between the stratum granulosum and the deeper layers, while others, the more specific, were located between the Malpighian layer and the derma, the papillae of the derma forming the floor of the vesicle.

In the formation of these vesicles Catrin traced the influence of a peculiar colloid degeneration of the epithelial cells, coagulation necrosis, and slight serous exudation. Beneath the vesicles the glandular structures usually showed cellular infiltration and the combination of these lesions produced the large elevated papules of "rougeole boutonneuse."

Finally Catrin described certain epithelial lesions which he regarded as specific of measles. The first of these consisted of the formation of colloid masses about the nuclei. These appeared first in the form of a single homogeneous globule at one side of the nucleus which increased in size, pushed the nucleus aside, or completely enveloped it, and eventually came to occupy a large portion of the cell or even distended its borders. By the fusion of these globules from adjoining cells large masses of colloid matter of mulberry form were sometimes produced. Associated with advanced stages of this colloid degeneration he noted increasing infiltration of the epidermis with lymphoid cells and coagulation necrosis of strands of cells producing at times a mass of necrotic epithelium isolated by lymphoid cells and a slight amount of serum. In the fresh condition the colloid globules ap-

<sup>1</sup> *Arch. de méd. expér.*, 1891, 3, p. 197.

peared like masses of gelatine and in sections of tissue they stained brick red with eosin.

Catrin mentioned that in one of his sections he observed in the pustules brilliantly stained ovoid bodies with large nuclei which he thought were evidently micro-organisms different in type from the schizomycetes.

Hlava,<sup>1</sup> 1906, excised Koplik's spots from the mucous membrane of the cheek in five cases of measles, and described two types of changes: (1) A parakeratosis or complete keratinization of the superficial layers with subsequent desquamation. This process he regarded as identical with the ordinary exanthem of measles; (2) A focal necrosis of the basal epithelial cells, with colliquation and formation of a vesicle or pustule which was surrounded by a superficial layer of keratinized cells. These circumscribed foci surrounded by a hyperemic zone correspond to Koplik's spots, which therefore represent a pustular measles eruption. From this report and from the observations on the present cases it would appear that the exact histology of Koplik's spots varies as does that of the general exanthem of measles. The term pustule does not accurately designate any of these lesions, since polynuclear leucocytes are scanty or absent in these foci.

In Gervelas<sup>2</sup> study of one case no specific changes in the skin were observed, but numerous cocci were found in the cutaneous structures.

In recent years additional interest would seem to attach to the minute histology of the lesions of the skin in measles owing to the fact that many studies of the lesions in other members of the group of exanthemata have appeared, especially of smallpox and lately of scarlet fever, in which the authors have described intracellular bodies which they believed to be protozoa.

It was from this latter point of view that I began in 1902 to collect and study material from cases of measles, and it is the object of this report to present the results of this study. The material has been collected slowly and with difficulty as the disease is not commonly fatal during the period of eruption. The first case was secured in 1902 at the Willard Parker Hospital through the kindness of Dr. W. H. Park, and several others were obtained at the New York Foundling

<sup>1</sup> *Časopis lékař česk.*, Prague, 1906, 45, p. 773, cit. in *Schmidt's Jahrb.*, 1906, 201, p. 242.

<sup>2</sup> *Allg. Wien. med. Ztschr.*, 1906, 51, p. 421.



Hospital through the co-operation of Dr. John Howland and Dr. Matthias Nicoll. The immediate incentive to the preparation of this report arose when Dr. Anna W. Williams of the New York Board of Health very kindly placed at my disposal prepared tissues taken from three patients during life. In this way the report comes to cover the study of nine cases of measles, on post-mortem material in six cases, and on Dr. Williams' material in three cases. For the assistance secured from all of these sources I take pleasure in expressing my thanks and obligation.

#### TECHNIC.

The tissues were fixed in various reagents, including Orth's fluid, Zenker's fluid,  $\text{HgCl}_2$ , and alcohol (50 per cent). Paraffin sections were stained with eosin and hematoxylin, eosin and methylene blue, eosin and polychrome methylene blue (Nocht's method), iodine green and fuchsin (Borrell's method), gentian violet, and Gram's method for bacteria.

#### DESCRIPTION OF LESIONS.

The changes observed in the skin in these cases constitute three rather distinct series, one of which is represented by a single case, one by two cases, while the remaining six cases fall in a group which seems to represent the more common lesions of measles.

*Case 1.*—Willard Parker Hospital. Autopsy six hours after death, by the writer. The body is of a well-nourished male child about four years of age. It is covered by a profuse general brownish maculo-papular eruption, in places over the chest nearly diffuse, elsewhere discrete. There is no appearance of scaling. Heart normal. In the lungs there are intense congestion and mucopurulent bronchitis extending to the finest bronchioles, but no areas of pneumonia. The liver is congested, not fatty, but shows a moderate number of minute focal necroses. The kidneys are pale and of slightly reduced consistence. There is moderate hypertrophy of the solitary and agminated intestinal lymph follicles. Spleen moderately congested.

The chief interest for the present report concerns the histological lesions of the skin, which proved to be of a most noteworthy character (see Figs. 1 and 2). The most striking feature of the epidermis is a marked thickening of the zone of keratosis which appears as a broad hyaline incrustation almost as wide as the Malpighian layer. In this zone appear numerous groups of imperfectly keratinized cells staining dark with methylene blue and representing the early stages of the branny desquamation of the disease. The exact steps in the outward progress of these groups of cells from the Malpighian layer to their observed position I have been unable to follow, nor do I find in the literature a satisfactory explanation of their mode of origin.

The cells of the Malpighian layer present a series of remarkable transformations which I believe to be quite unique and specific. They may best be traced in the drawing (Plate I, Fig. 1). Almost the entire mass of cell protoplasm and nuclei is broken up into a series of curious figures which includes ring-shaped bodies, homogeneous spherical globules, crescent-shaped masses, and fragments of highly irregular outline. The nuclei are intact but homogeneous, or extensively vacuolated, or broken into fragments which sometimes show vacuoles in geometric arrangement, while the nucleoli are absent or displaced, or shrunken or vacuolated. The cells of the hair follicles and sebaceous glands are similarly affected. Between the epithelia are occasionally seen isolated elongated homogenous cells in coagulation necrosis.

The extent and variety

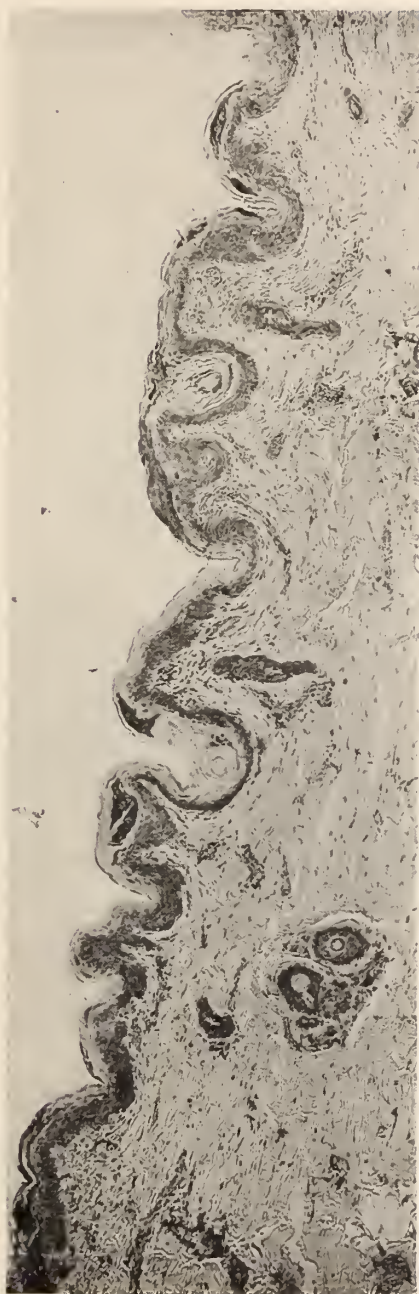


FIG. 1. Diffuse hyperkeratosis and abundant scale formation in measles (Case 1). Bichloride, eosin, and methylene blue. Specimen shows continuous thick layer of zone of keratosis, in places involving nearly the whole epidermis and containing very numerous dark-staining adherent scales.

of these cellular changes exceed anything I have seen in variola or scarlatina, but in some respects they resemble the peculiar intracellular structures described in these latter diseases. It is difficult to find a phrase which will suitably designate this series of changes but they seem to fall into the general class of degeneration, coagulation necrosis, and disintegration of epithelial cells such as in some degree

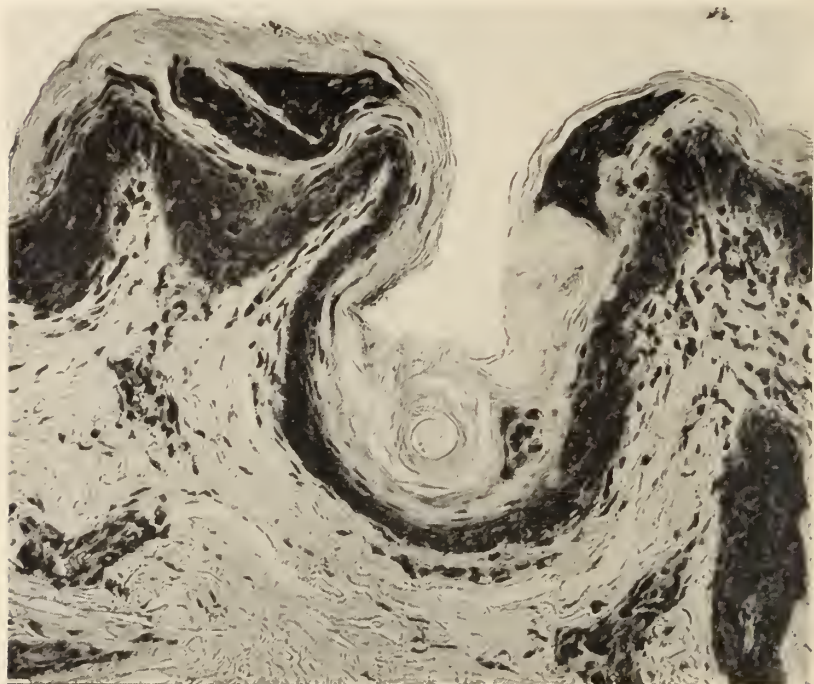


FIG. 2.—The same as Fig. 1, in higher magnification.

characterize all the exanthemata. They would thus seem to represent the reaction of the epithelium to the toxic agents of the disease, and their occurrence in such striking form demonstrates that in measles as in variola and scarlatina the epidermis may react in a specific manner. In a considerable number of other specific diseases of the skin I have been unable to find similar alterations of epithelial cells.

In the mucous membranes the lesions were less striking and peculiar. In the pharynx there is slight general edema of the sub-

mucosa and very numerous collections of round cells. The epithelium shows a marked desquamative process with definite erosions but no distinct ulcers or focal necroses. In the transitional epithelium of the larynx the superficial flat cells are often in coagulation necrosis or keratinization and there is a bulky growth of cocci in and about these superficial cells. The submucosa of the larynx and trachea shows extensive diffuse infiltration by round cells.

*Case 2.*—(No. 1897.) Hemorrhagic measles. New York Foundling Hospital. Patient of Dr. Nicoll. Age  $2\frac{1}{2}$  years. Died on the fourth day from uncomplicated measles. A complete autopsy was not permitted but portions of skin from the back and abdomen were obtained 12 hours after death, and placed in 50 per cent alcohol.

The lesions observed in this material were striking and peculiar. They consisted chiefly in the formation of perinuclear vacuoles, single or multiple, large and small, sometimes displacing the nucleus to one side, sometimes completely surrounding the nucleus. Occasionally the vacuoles from adjoining cells were fused and often an elongated vacuole extended some distance between the cells, pushing the cytoplasm ahead as a tongue-like protrusion. These vacuoles contain a faintly basic-staining homogeneous material which is recognizable also by its refractive properties. In the vacuoles also are almost invariably found one or more densely staining basophilic bodies, the origin and nature of which it is difficult to determine. Many of these are very minute, some are large, irregular globules. Definite ring shapes appear and it is possible that some of the minute bodies are parts of larger ring-shaped structures. Some resemble biscuit-shaped diplococci, others resemble elongated pear-shaped cocci. A marked lack of uniformity in size and shape leaves a very confusing impression with the observer. The bodies are not limited to the vacuoles but many are found scattered in the cytoplasm, and the intercellular spaces, especially between the basal cells, may be closely filled with them. Neither are they limited to the epithelial layers, but in the subepithelial lymph spaces and capillaries very numerous collections are found, and they appear also in and about all the epithelial structures of the derma. They are much more numerous than is indicated in the sketch (Plate 1, Fig. 2). They are Gram-negative, and stain densely with gentian violet, and distinctly with strong eosin.



After numerous attacks upon the question I have been unable to reach a satisfactory conclusion as to the nature of these bodies. They are not bacteria. Their numbers and distribution might suggest to some a protozoon hypothesis, as many of the forms show resemblance to the piroplasms. I am more inclined to regard the appearances as the result of coagulation of an albuminous exudate or degenerative product derived from the epithelium. In a considerable series of other diseases of the skin examined for control I have found very similar bodies in and between the epithelial cells in one acute case of *pityriasis rosea*. This disease bears some clinical resemblance to measles.

*Case 3.*—(No. 1966.) New York Foundling Hospital, May 16, 1904. Patient of Dr. Nicoll. Age six years. Died on the fourth day of well-marked maculo-papular eruption with symptoms of broncho-pneumonia. Autopsy by the writer, 36 hours after death.

Anatomical diagnosis: Broncho-pneumonia. Ulcerative stomatitis and pharyngitis. Catarrhal bronchitis. Acute degeneration of liver and kidneys. Abundant fine papular exanthem of measles.

Portions of skin were taken from the trunk, arm, and leg, and hardened in Zenker's fluid. The lesions observed in the skin consisted in the appearance of large vacuoles about the nuclei of cells of the Malpighian layer, the presence of isolated necrotic cells in this layer, and the occurrence of focal points of necrosis of epithelial cells leading to the formation of small vesicles or pustules. The vesicles were often found in connection with similar changes in the sebaceous glands and hair follicles (Figs. 3, 4), these latter structures being nearly always extensively affected.

The minute changes in the necrotic foci are detailed in the colored sketch (Plate 2). There is a small central cavity containing granular coagulum and detached necrosing epithelia. The next adjoining cells show various stages of coagulation necrosis. Beyond these come some isolated necrotic cells, others with large perinuclear vacuoles, while some are but slightly altered. A great variety of peculiar structures is found in the cytoplasm of the degenerating cells, in the perinuclear vacuoles, and between the loosened cells. Most of these are represented in the sketch and it may be said of them that very similar bodies may be found in variolous lesions and in the experimental necrosis of epithelium produced by diphtheria



toxin. Others resemble the structures described in and about the cells in Case 2. In the sweat glands especially there are very numerous perinuclear vacuoles containing minute granules and rings identical with those seen in Case 2 and sketched in Plate I, Fig. 2.

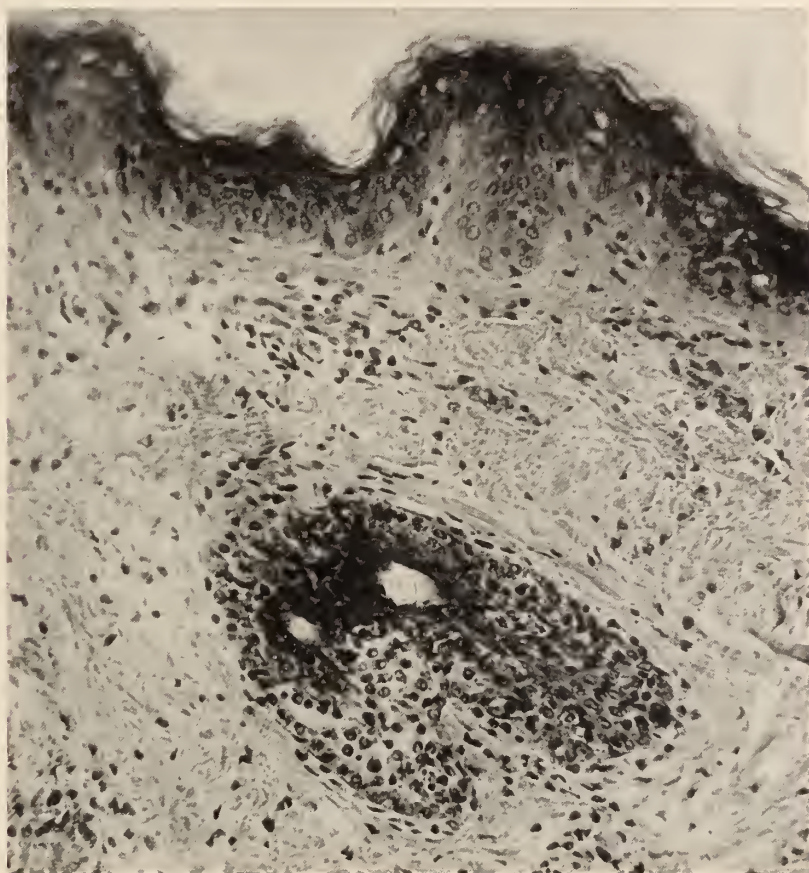


FIG. 3.—Hair follicles of measles (Case 3). Zenker's fluid, eosin, and methylene blue. Specimen shows hydropic vacuoles in epidermis; edema, and increase of large round cells of derma; edema, and various stages of degeneration of cells of hair follicle.

There is a uniform increase in the number of large round cells in the derma, and these seem to be divided among exuded large mononuclear leucocytes and multiplying endothelia. The vesicles are usually capped by a thickened layer of hornified scales. Bacteria could not

be identified in the epithelial layers, but in this as in other cases the more superficial scales always contained a number of cocci.

Mucous membranes. The buccal, pharyngeal, and entire length of the respiratory tract was carefully examined for gross, micro-



FIG. 4.—Hair follicles of measles (Case 3). Specimen shows extension of lesion of hair follicle into epidermis.

scopical, and minute intracellular changes. There were found the results of a universal intense catarrhal inflammation with marked desquamation of cells, and pronounced infiltration of the submucosa with large and small round cells. In the larynx, trachea, and bronchi the desquamation of lining cells produced occasional erosions down

to the membrana propria or entering the mouths of mucous glands. In the mouth and pharynx there were isolated necrotic cells, general edema, and many small focal necroses in the epithelium, comparable to those of the skin, giving small, crater-like ulcers. The vessels beneath these ulcers were always intensely congested, or filled with densely packed red-cell thrombi. These lesions seem to fall in the class of Koplik's spots (Fig. 5). At a few points in the pharyngeal mucosa there were larger and deeper ulcers covered with fibrinous

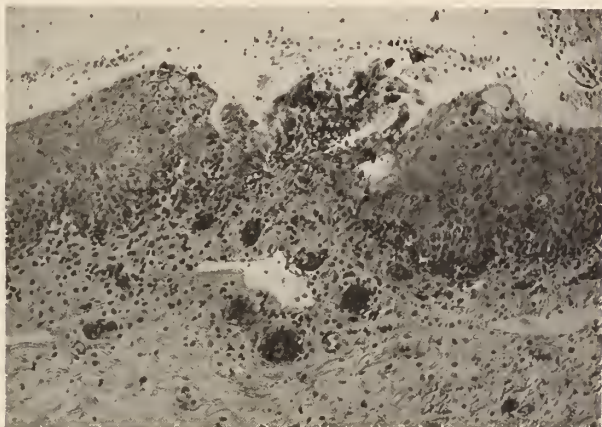


FIG. 5.—Focal ulcer of buccal mucosa in measles (Case 3). Specimen shows a small necrotic focus involving the whole layer of epithelium. The slough is adherent. The submucosa contains small vessels gorged with blood and some round cell infiltration.

membrane (Fig. 6). Throughout the lungs there was intense congestion in interstitial exudative and productive bronchitis and beginning broncho-pneumonia. The liver showed congestion, granular degeneration, and foci of intense fatty degeneration. The kidneys were intensely congested and the tubule cells in marked granular degeneration.

*Case 4.*—New York Foundling Hospital, May 17, 1904. Patient of Dr. Nicoll. Died in the acute eruptive stage of measles, with terminal convulsions. The exact day of the disease was not determined. A complete autopsy was not permitted but portions of skin from the arm and trunk were obtained, which showed a fine maculo-papular eruption.

Microscopical examination showed changes in the skin which were identical in character with those of Case 3. The focal necroses were less numerous, but isolated necrotic cells, edema, and perinuclear vacuolation were rather more noticeable.



*Case 5.*—(1895.) New York Foundling Hospital, February, 1904. Patient of Dr. Howland. Age  $4\frac{1}{2}$  years. Died in the early stage of desquamation of measles with symptoms of broncho-pneumonia.

Anatomical diagnosis: Broncho-pneumonia. Acute degeneration of liver and kidneys. Congestion of spleen. Acute pharyngitis and laryngitis. Branny desquamation of skin.



FIG. 6.—Superficial ulcer and diffuse necrosis of lower pharyngeal mucosa (Case 3). There is a large, partly denuded area of epithelium, the necrosis extending at one point into the submucosa. At the left the basal cells show early necrosis. The edge of a second ulcer appears on the right. The blood vessels are distended, and there is edema, fibrinous exudate, and round-cell infiltration of the submucosa.

Microscopical examination: Apart from the presence of many loose desquamating scales there are few changes in the cutaneous epithelium. A good many cells showed mitotic nuclei and a few homogeneous perinuclear bodies were found, but the usual hydropic vacuoles and isolated necrotic cells or necrotic foci were missing. There is considerable congestion of the skin, and some increase of plasma cells, endothelia, and round cells about the basal epithelium vessels and glands. The skin in this case therefore represents the healing stage of measles.

**Pharynx.** There is considerable edema and increase of round cells beneath the epithelial layer and some edema and hydropic degeneration of the epithelium. These changes tend to be focal. At one point a definite area of superficial necrosis covered by fibrin was found. **Larynx and trachea** show active catarrhal inflammation with extensive desquamation of lining cells and much increase in the round cells beneath the epithelium. At some points near mouths of mucous glands there are superficial erosions. In the bronchi and bronchioles the catarrhal inflammation becomes more intense, there are advanced hydropic degeneration of lining cells and much desquamation. To these changes is finally added, as one reaches the pulmonary parenchyma, an interstitial exudate containing many round cells, and the lesion eventually passes into definite broncho-pneumonia with considerable diffuse exudation into the alveoli. At no point were specific lesions of the epithelial cells observed. The liver shows advanced granular and fatty degeneration, with focal areas of intense fatty degeneration. The kidneys show acute exudative nephritis. The spleen is extensively congested.

*Case 6.*—New York Foundling Hospital, February, 1904. Patient of Dr. Nicoll. Died in the stage of desquamation with symptoms of broncho-pneumonia. The material consisted of portions of skin only, a complete autopsy not being obtained.

The skin over most of the areas examined appears normal. The scales are numerous and loose. In a few small areas there is excessive keratosis involving much of the Malpighian layer and here many cells show the peculiar changes described in Case 1. In many hair follicles and in all the sweat glands these peculiar changes were very marked.

*Case 7.*—(501.) The material from this case, furnished by Dr. Williams, consisted of several pieces of skin snipped off under cocaine from a child of three years, on the second day of a well-marked measles eruption of maculo-papular type. Fixation, Zenker's fluid. Stains by eosin and hematoxylin, methylene blue, gentian violet, and Gram's method.

**Microscopical examination:** There is general edema of the Malpighian layer and of the subepithelial connective tissue. There are many isolated necrosing homogenous epithelial cells and a few typical focal necroses, some of which are connected with subepithelial foci of round cells or altered sebaceous hair glands. Perinuclear vacuoles are prominent and there are many mitoses. A few flattened mononuclear leucocytes lie between the epithelia. The endothelium of the capillaries is swollen, proliferating, and many large round cells are packed in and about the capillaries. In many of the vacuoles of basal and other cells, in the lumina of capillaries, in the endothelium, in the large perivascular cells, and in the cells of the sweat glands, are basic-staining granules, rods, or rings similar to those described in Case 2. They are more prominent after methylene blue and still more so after gentian violet, and they are Gram-negative. Lesions in this case seem to be intermediate between those of Cases 2 and 3.

*Case 8.*—(499.) Material supplied by Dr. Williams, from a patient five years old, on the second day of eruption, from the skin of thigh.

The description of this case is very similar to that of the preceding one, but definite focal necroses were not found, rather numerous polynuclear leucocytes were present in the capillaries, and the peculiar granules and rings in vacuoles and cell bodies were much less numerous.

*Case 9.*—(502.) Material supplied by Dr. Williams. Child of eight months, third day of eruption, skin of chest.

\* Description similar in all respects to the one preceding, except that a very few early foci of necrosis were observed.

## EPICRITICAL.

The findings in the preceding cases indicate that the usual cutaneous lesions of measles are those enumerated under Case 3, appearing also in Cases 4 and 7, and consisting in: (1) focal necroses with the formation of small vesicles; (2) isolated necrotic epithelia; (3) diffuse perinuclear vacuolation of cells of epidermis and of dermal glandular structures; and (4) congestion, edema, swelling, and proliferation of endothelial cells, and moderate increase of large round cells (see Fig. 3).

In a case of hemorrhagic measles (Case 2) the focal necroses were wanting but all the other lesions mentioned above were present, and, in addition, in the vacuoles of the epithelium in all situations, and in and about the capillaries and lymph spaces were very large numbers of peculiar granules or ring-shaped structures. The nature of these bodies is left undetermined but the most probable hypothesis is that they represent a coagulated albuminous material derived from the blood and from degenerating epithelium. Apparently similar bodies in smaller numbers were noted in other cases (Cases 7 and 8).

In a case of nearly confluent measles eruption focal necrosis and perinuclear vacuolation were absent, and instead one finds hyperkeratosis and a peculiar form of degeneration of the Malpighian cells marked chiefly by the homogenization and fragmentation of cell bodies and nuclei with the appearance of very many peculiar geometrical and vacuolated structures. Such changes not having been found in other diseases, they may for the present be regarded as specific for certain cases of measles. Similar changes of less general distribution were found in a second case. Therefore at least three rather distinct series of histological lesions may be regarded as belonging to the eruption of measles. The commonest lesion encountered in the present study, focal necroses with formation of vesicles, appears to be essentially the same as those described by Catrin but very imperfectly recognized by previous writers. The deep "phlyctenes" of Catrin did not appear in the present series of cases. The exudation and infiltration in and about the hair follicles and sebaceous and sweat glands described by the older writers were present in some cases but absent in others, so that these changes can probably not retain the specific importance in the measles process which has been formerly attributed to them.

The perinuclear "boules colloïdes" of Catrin seem to be identical with the faintly staining homogeneous material in the perinuclear vacuoles of the present descriptions, but the former stained intensely with eosin, while in the material of the present study it proved faintly basophile. The larger conglomerate masses of colloid material described by Catrin seem to be referable to groups of necrotic epithelium, or else they were not encountered in my cases.

Since no single type of cellular changes is invariable in measles and since the lesions observed vary so widely, the question may arise whether the clinical diagnosis of measles as at present applied may not really cover more than one infection. This question seemed unavoidable when the peculiar changes observed in a case of hemorrhagic measles (Case 2) were found nearly duplicated in a case of *pityriasis rosea*. However, the clinical features of measles are extremely characteristic, and in view of this fact and of our ignorance of the etiological agent, it would seem that the occurrence of quite different epithelial changes would not warrant one in seriously urging that several infections are now passing under the term of "measles." In variola, primary hemorrhagic, diffuse or confluent, and discrete pustular forms, the minute changes in the epithelial cells are quite different, but the disease itself is almost certainly a single infection.

As with the other exanthems, measles is characterized by a severe inflammation of the pharynx and respiratory tract. Except for its very frequent complication with broncho-pneumonia, it does not appear to be as severe as that of scarlatina, or nearly as serious as that of variola, but, as with both of these maladies, it antedates the exanthem. In the present cases it consisted of an intense catarrhal inflammation with marked desquamation of cells, superficial erosions, minute focal ulcers, and extensive subepithelial infiltration with round cells. As in smallpox and scarlet fever the peculiar epithelial changes and structures seen in the skin are largely or wholly missing in the mucous membranes. If there is anything specific in these lesions of the mucous membranes it is the extensive subepithelial infiltration with round cells, which often shows a focal distribution, and the focal necroses, and it has seemed to me that the occurrence of the former probably determines the latter lesion in skin, pharynx, and respiratory tract. Koplik's spots seem to be one expression of this focal character of the process.



The lymphocytosis of measles is fully explained anatomically by the intense congestion and hyperplasia of the lymphoid tissues. The frequency of tuberculous sequelae in measles may perhaps be referred to the unearthing of buried tubercle bacilli from these lymph nodes. The internal organs in measles seem to exhibit little that is specific of the disease. In the livers examined there was considerable granular and fatty degeneration, and these changes were more intense in certain foci reaching at times the condition of the focal necroses described by Freeman.<sup>1</sup>

From the study of the present cases one may perhaps be warranted in theorizing briefly concerning the general nature of the virus of measles. In the writer's opinion all the indications drawn from these cases point to the existence in measles of an infection by an actively multiplying micro-organism of the class of bacteria. Although one finds in the epithelial cells many structures which bear some resemblance to protozoa and the origin of one group of these structures could not be satisfactorily determined, yet the general characters of the disease, and especially the widespread occurrence of very acute degeneration and necrosis of epithelial cells, all suggest that measles is referable to infection by a bacterium which produces an active toxin having special affinity for superficial epithelial cells.

#### DESCRIPTION OF PLATES 1 AND 2.

##### PLATE 1.

FIG. 1.—Confluent measles (Case 1). Fixation: Bichloride. Stain: Borrel's iodine green and fuchsin. Specimen shows excessive zone of keratosis, isolated necrotic cells, and a variety of curious bodies resulting from degeneration, vacuolation, or fragmentation of nuclei and cell bodies.

FIG. 2.—Hemorrhagic measles (Case 2). Fixation: Alcohol 50 per cent. Stain: Eosin and methylene blue. Specimen shows perinuclear vacuoles containing basophilic granules, globules, rings, and elongated rods. Similar structures are seen in the cytoplasm, between the cells, and within the subepithelial vessels.

##### PLATE 2.

Common vesico-papule of measles (Case 3). Fixation: Zenker's fluid. Stain: Eosin and methylene blue. Specimen shows a small central area containing loose necrotic epithelia and serous exudate, surrounded by coherent necrotic cells. Beyond are isolated necrotic cells, perinuclear vacuoles, and peculiar perinuclear and cytoplasmic structures.

<sup>1</sup> *N. Y. Med. Jour.*, 1898, 54, p. 136; also *Studies from Dep't of Path., Coll. Phys. and Surg., N. Y.*, 1899, 7.



PLATE I

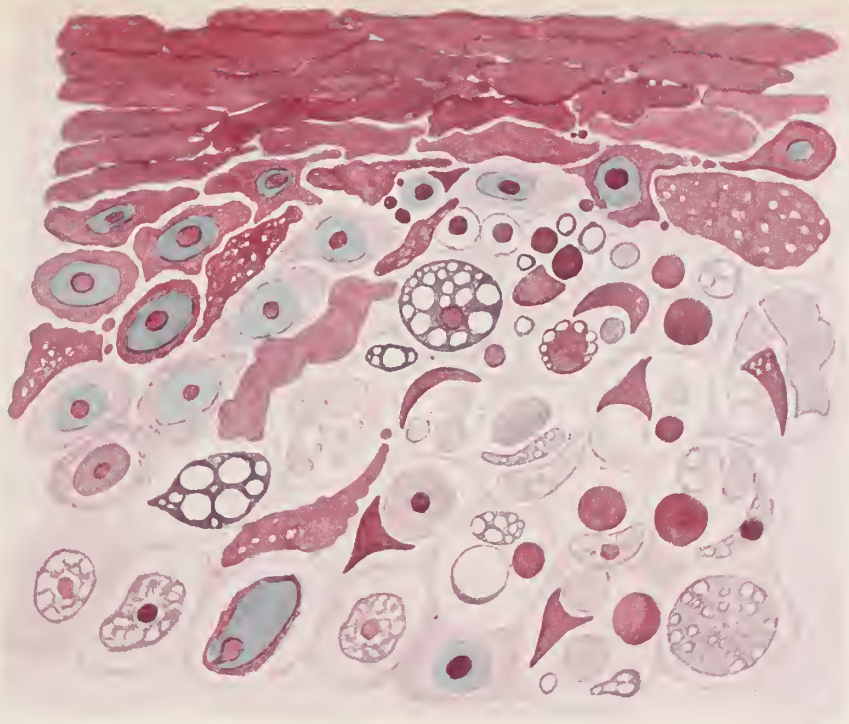


FIG. 1

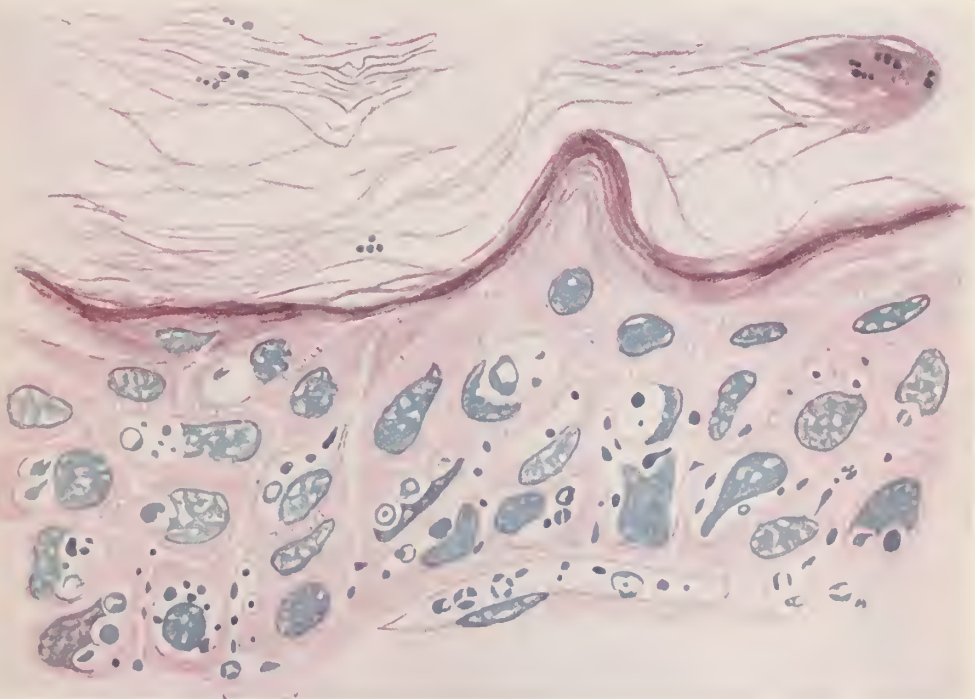
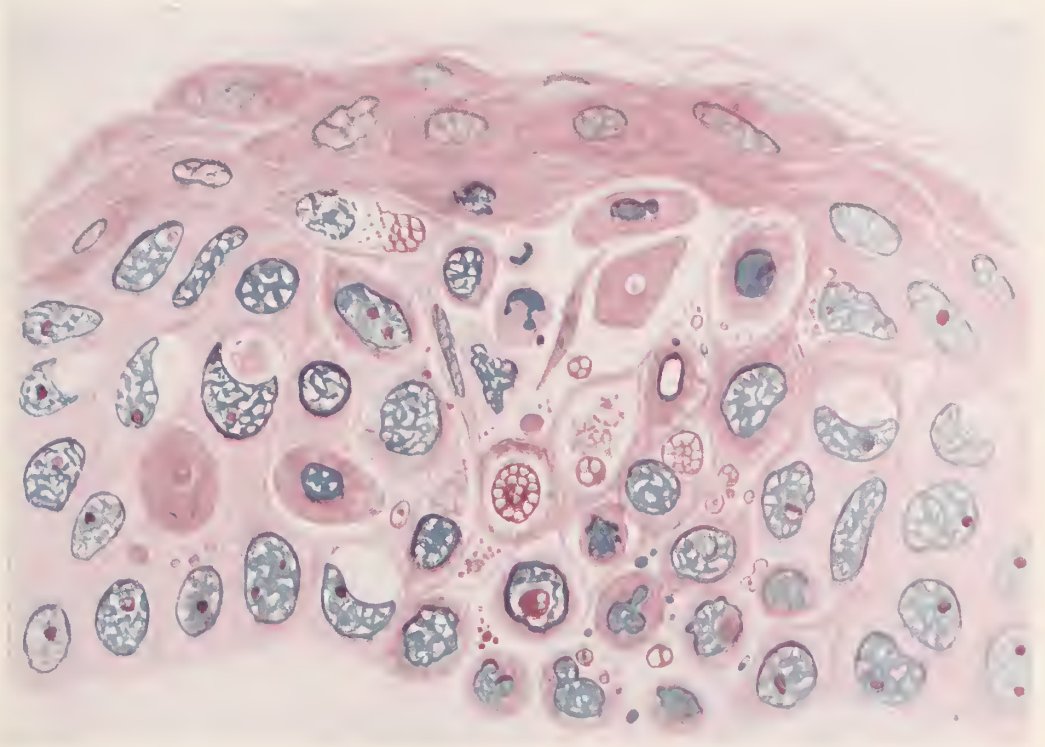


FIG. 2



PLATE 2





# THE NUMERICAL RELATIONSHIP OF *TREPONEMA PALLIDUM* TO CERTAIN PATHOLOGICAL TYPES OF CONGENITAL SYPHILIS.\*†

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## SYNOPSIS.

### INTRODUCTORY.

RESULTS OF THE ROUTINE EXAMINATION OF ALL FETAL AND INFANTILE MATERIAL FOR *Treponema pallidum*.

CHRONIC INFLAMMATORY CHANGES WHICH MAY LEAD TO ERRORS IN DIAGNOSIS. THE ESSENTIAL PATHOLOGICAL CHANGE IN SYPHILIS.

THE NUMERICAL RELATIONSHIP OF *Treponema pallidum* TO NEONATAL LUES:

A. Cases showing chronic lesions:

1. Born living.
2. Still-born.

B. Cases showing no characteristic lesions:

1. Born living.
2. Still-born.

EXPLANATION OF THE VARYING NUMERICAL RELATIONSHIP.

THE CAUSES OF DEATH IN CONGENITAL LUES.

GERMINAL INFECTION.

SUMMARY.

## INTRODUCTORY.

IN a previous paper<sup>1</sup> I have reviewed the facts favoring the etiological relationship of *Treponema pallidum* to syphilis. Further work has offered nothing which would lead to a change in the conclusion reached at that time, namely, that *Treponema pallidum* must be considered the cause of lues. The classification of neonatal syphilis attempted in this paper is dependent entirely upon the establishment of the causal relationship of the organism to the disease, since the determination of the luetic nature of certain of the cases is not possible without the demonstration of the organism.

### RESULTS OF THE ROUTINE EXAMINATION OF ALL FETAL AND INFANTILE MATERIAL FOR *TREPONEMA PALLIDUM*.

In working with material from fetuses and infants supposedly syphilitic I was early struck by the comparatively large number of

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<sup>1</sup> *Jour. Cutaneous Dis.*, 1907, 25, p. 429.

cases in which the clinical diagnosis was hereditary lues, in which there were certain pathological changes that might be considered syphilitic, but which failed to show *Treponema pallidum*. It seemed necessary, therefore, in order to arrive at a proper diagnosis, to use the Giemsa and the Levaditi methods as routine procedures on all fetal and infantile material which came to my hands. Such a routine technic soon brought out a second striking fact, namely, the presence of *Treponema pallidum* in cases which show none of the pathological changes that permit one to make a histological diagnosis of syphilis.

Working in this way I was led to make the statement, in the paper referred to above, that the clinical diagnosis of hereditary syphilis is wrong in a rather large proportion of cases. Further work forces me to reaffirm this belief, but in so doing I have no wish to wound the feelings of the clinician. For the proper recognition of any condition the clinician is dependent, ultimately, upon the pathologist. If the latter errs, the clinician must necessarily follow along the mistaken path blazed out for him. Too often the clinical diagnosis of congenital lues is based upon a suspicious parental history, desquamation of the superficial epidermis of the palms and soles, congestion and eruption of the skin of the buttocks, a palpable spleen, inflammation about the finger nails, rhinitis, and possibly ulceration of the buccal mucosa. That such manifestations can and do often occur cannot be doubted, but that any or all of them are evidences only of syphilis and that they are not met with in non-specific conditions must be doubted. In by far the greater part of my material I have had to deal with illegitimate children, and one is apt to take a syphilitic history on the part of the parents for granted in such cases. When a woman gives birth to an illegitimate child it is quite evident that she must "admit exposure," even if she does "deny lues." While women who practice more or less promiscuous prostitution certainly do run very strong chances of infection, the latter does not necessarily occur in every case, and a diagnosis which rests upon the mere possibility of contagion is apt to be based upon insufficient grounds.

In the liability to be misled by the parental history and the clinical manifestations the clinician and the pathologist react upon



each other. The latter, having a clinical diagnosis of lues before him, confirms it if he finds chronic fibrosis of the spleen, chronic interstitial changes in the lungs, enlargement of the liver, or more or less well-marked irregularity of the ossification lines. The clinician, having been confirmed in his diagnosis, is not to be blamed if he considers all other similar cases syphilis. As long as the histological diagnosis is based upon the presence of changes of a chronic inflammatory nature, mistakes will be made in two directions. Firstly, there are undoubtedly cases which do not show such changes but which certainly are syphilis, and secondly, there are cases which do show such chronic inflammatory lesions but which certainly are not syphilis. Now that the etiological agent concerned in the disease is known, the making of a diagnosis can be approached with greater scientific precision than has heretofore been possible, and I feel sure that the histopathology of syphilis will undergo even greater revision than has that of tuberculosis.

When there is present the classical pathological complex: pneumonia alba, fibrosis of the spleen, chronic interstitial hepatitis, proliferation of the interstitial tissue of the kidneys, osteo-chondritis, and skin lesions often of a bullous or pemphigoid character, there is little chance for error in the pathological diagnosis. But when only one or several of these changes are found the matter becomes more difficult.

#### CHRONIC INFLAMMATORY CHANGES WHICH MAY LEAD TO ERRORS IN DIAGNOSIS.

A chronic interstitial pneumonia, strikingly like the pneumonia alba of Virchow, is extremely common in children dying within the first two or three months after birth. There is the same fibroblastic proliferation of the septa, leading to swelling and desquamation of the alveolar epithelium and to final complete obliteration of the air spaces, and there may be just as marked a degree of lymphoid infiltration. That the only essential and characteristic change in syphilis is the vascular involvement will be discussed later. When the connective tissue proliferation in the lung becomes marked, the vascular changes form a very small part of the microscopic picture, and may, indeed, become impossible of recognition in an advanced pneumonia

of undoubted syphilitic origin. It is true that in the non-specific interstitial pneumonias referred to here the pathological change begins in the posterior portions of the lungs and may remain limited to them. It is not unusual, however, to find practically all of the lower lobe and a considerable portion of the rest of the lung involved. The syphilitic pneumonia likewise begins in the lowermost regions of the lungs, and need not involve a very great amount of lung tissue. The non-specific change under discussion occurs in infants who do not thrive and in whom there is well-marked hypostatic congestion. Whether the pathological change is produced in a purely mechanical manner by the chronic congestion alone, or whether it may possibly be due in some cases to bacterial infection I am not in position to decide at the present time. That the condition is a chronic inflammatory process is certain. The mechanical factor, the hypostatic congestion, also acts in the syphilitic pneumonias, and I see no reason why it may not account for a great part of the interstitial change which occurs in pneumonia alba. Unless vascular involvement takes a fairly prominent part in the picture it becomes impossible to decide, upon purely histological grounds, as to the specific or non-specific character of the pneumonia.

The determination of the syphilitic nature of a fibrous lienitis in any given case may also be a matter of considerable difficulty. Moderate enlargement of the spleen, due to an increase in the amount of the stroma and to endothelial proliferation, may happen independently of syphilis. In some cases the proliferative change is at the expense of the lymphoid tissue and may lead to almost complete disappearance of the Malpighian bodies. In other cases the latter also are hypertrophied—all the elements of the spleen seem to be equally involved. Of course, if characteristic arteritis and phlebitis are marked, the proper diagnosis is easily made. But even in cases in which *Treponema pallidum* is present in the spleen the vascular involvement may be masked or hidden by the diffuse stroma proliferation.

As to the difficulty in making a histological diagnosis of syphilitic osteo-chondritis so experienced an investigator into the pathology of bone as Schmorl has given testimony. When considered in connection with changes in the other tissues and organs one, as a rule,



does not hesitate to affirm the specific nature of bone lesions in congenital lues. And yet, when the bone lesion is studied by itself, it is extremely difficult to say just what change is characteristic of syphilis. I have several times seen, more particularly in fetuses born dead near the end of pregnancy, in cases in which *Treponema pallidum* could not be demonstrated, changes in the long bones which manifested themselves by an opacity and thickening of Guerin's line recognizable by the naked eye. Microscopically, there is irregularity of and variation in the thickness of the zone of preliminary calcification, irregularity of the zone of advancing ossification, and an increase in the number of lymphocytes of the marrow. The proper interpretation of changes of this nature is a matter of difficulty. If one believes, with Schmorl, that rachitis is a disease of post-fetal life, the change is not due to rickets, because all the cases in which I have observed it have been still-born fetuses or infants dying within a short time after birth. Moreover, I have not been able to detect the overproduction of uncalcified osteoid tissue, a change which, according to Schmorl, must be present in order to permit one to make a diagnosis of rickets. A priori, I can see no reason why the imperfect and irregular ossification that characterizes this disease might not begin during fetal life. "Fetal rickets" has been described, but the majority of competent observers deny that it has anything to do with the rachitis of post-fetal life. Under the designation, "fetal rickets," are included cases of chondrodystrophia fetalis and of osteogenesis imperfecta. Both conditions are associated with skeletal deformities. These were not present in my cases. As stated above, the changes noted consist of variation in the thickness of the zone of preliminary calcification, irregularity of the zone of advancing ossification, and an increase in the number of marrow lymphocytes, without any noticeable overproduction of osteoid tissue. The lesion seems to be either a very early chondrodystrophia fetalis or, possibly, a true rachitis beginning during intra-uterine life.

In discussing the chronic interstitial pneumonias the statement was made that in a true syphilitic pneumonia alba it is practically impossible to say how much of the change is due to syphilis and how much to the factors which produce the non-specific type of pneumonia. A difficulty of the same kind is encountered in attempting

to explain a syphilitic osteo-chondritis. That *Treponema pallidum* may be present in the area of pathological change is known. And yet, in one of my cases of fetal lues which shows numerous organisms, the lower end of the femur shows no treponemata, although there is irregularity of calcification and ossification, and an increase in the number of lymphocytes. It is possible that the bone lesion in this case is not really syphilitic, but is an example of the non-specific change just discussed.

Chronic interstitial inflammatory changes in the liver and the kidneys, not associated with syphilis, are rare. There have been no such cases in my own material.

One other condition, which may, perhaps, be termed a chronic lymphadenitis, deserves mention, since it might lead to error in the histological diagnosis of lues. I have seen two such cases, one an infant dying a few hours after birth, the other several days after birth. In both syphilis was suspected, but no treponemata could be found, either by the Giemsa method on smears or by the Levaditi impregnation method. In the mesenteric and retro-peritoneal lymph glands the stroma is markedly prominent, the sinuses and capillaries are dilated, and lymphoid cells, particularly small mononuclears, are present in very small numbers. Furthermore, the intestinal lymphoid tissue shows similar changes, and the number of lymphocytes in the bone marrow and in the spleen is smaller than normal. In speaking of these changes as chronic lymphadenitis the term is used for the sake of convenience. I am not convinced that they are inflammatory in nature. One must consider the possibility of a fetal mal-development of lymphoid tissue throughout the body. An enumeration of the lymphoid cells of the circulating blood might help one in arriving at a proper conclusion as to the nature of the condition. Unfortunately, this was not done in the two cases here considered.

In a nearly full-term still-born fetus the pancreas shows a marked increase in interstitial tissue. The latter is rich in young connective tissue nuclei and in lymphocytes, and newly formed blood vessels are fairly numerous. The spleen in this case shows some increase in the amount of stroma. *Treponema pallidum* could not be demonstrated.

I have attempted to set down changes, most of them of a chronic inflammatory nature, occurring in various organs. The changes are such as to lead to difficulty in making a histological diagnosis of congenital lues. Such experience as I have had, based upon autopsies of over one hundred fetuses and newly born infants, makes me extremely skeptical as to my own ability to state definitely that a given chronic inflammatory change in a given single organ is due to syphilis. I am unwilling to consider the lesion specific unless *Treponema pallidum* can be shown to be present by the silver nitrate method. It may be argued that, in general, one would not make a histological diagnosis of syphilis, based upon changes limited to any one organ. I am willing to admit that there is little chance for error if the lungs, liver, spleen, and bones show the changes that are considered characteristic. But if one demands this complex for the establishment of a diagnosis he will fail to recognize the luetic nature of a considerable proportion of cases. Some of these will show no change, a group to be considered in more detail later. In other cases there is pathological change, but it is limited to a single organ, and often is slight in extent.

As to the proper explanation of the causation of the non-specific changes under discussion, I am not yet ready to hazard a very definite opinion. The question is the subject of other investigations already begun. When these changes occur in infants born alive and dying within the first few weeks, the clinical history will show that one is dealing with children who have never thriven and who have not received the proper returns from their food. Their physical condition is that of marasmus. The interstitial pneumonia in such cases may find an adequate explanation in a chronic hypostatic congestion. The interstitial change in the spleen, the slight abnormalities at the ossification lines of the long bones, and the marked degree of parenchymatous degeneration which may be present in the liver and the kidneys may express, in part, the disturbed metabolism of the infant. Faulty assimilative processes may also help explain those bone lesions present in still-born fetuses in whom *Treponema pallidum* cannot be found.

It may be argued that some of these conditions, which are here grouped together as non-specific, are really syphilitic and that the

organisms have disappeared from the tissues. This seems improbable. So long as necrosis is not marked, the organisms can readily be demonstrated, and their presence in a non-necrotic lesion is the only criterion of the luetic nature of the inflammation.

#### THE ESSENTIAL PATHOLOGICAL CHANGE IN SYPHILIS.

If the chronic interstitial inflammations discussed above cannot be considered characteristic of syphilis, is there any histological change which may be considered specific? Among the host of investigators who have studied the histopathology of acquired syphilis there is unanimity in the opinion that the primary and essential change is vascular involvement. There has, of course, been a considerable amount of controversy, but this resolves itself into discussion as to whether the change begins in the lymphatics, the veins, or the arterioles, and, secondly, as to whether the lesion is primarily an endo-, a meso-, or a perivascular one. The study of the localization of *Treponema pallidum* in the organs and tissues of hereditary lues has added much of importance to our knowledge of the pathogenesis of the disease. The silver nitrate method shows that, in spite of the more or less widespread distribution of the organisms, the chief and characteristic localization is a perivascular one. Moreover, examination of suitable material shows that the earliest pathological lesion is likewise perivascular. The earliest change is a swelling of the endothelium of the perivascular lymph spaces. This is soon associated with beginning connective tissue proliferation and with a moderate degree of lymphoid infiltration. In this early stage eosinophilous leucocytes may be present in considerable numbers. The lesion begins with the localization of *Treponema pallidum* in the perivascular lymphatics. The organisms apparently leave the lumina of the blood vessels as rapidly as possible, and then multiply in the lymphatics. This characteristic localization of the organisms occurs not only about the blood vessels, but also in the peribronchial lymphatics and in those about the bile ducts. *Treponema pallidum* may be so numerous in these situations that, in silver nitrate preparations, the blood vessels, bronchi, and bile ducts may be seen with magnifications as low as one hundred diameters as spaces heavily outlined in black. From the perivascular lymphatics the organisms

spread in two directions. They penetrate the wall of the vessel, being less numerous in the media than in the adventitia, and still less numerous in the intima. They also migrate away from the vessel and invade the tissues about it. This spread of the organisms goes hand in hand with a spread of the inflammatory process, which manifested itself first as a moderate proliferation and infiltration in the adventitia of the vessel. The fixed cells of the media and of the intima proliferate, the endothelium undergoes first hypertrophy and then hyperplasia, and lymphocytes make their appearance. The vascular inflammation may reach a grade so extreme that the tissues of the vessel wall become widely separated and almost completely hidden by a dense infiltration of lymphocytes. So marked an infiltration of the vessel wall is, however, very infrequent. The spread of the organisms into the tissues about the vessels is associated with a chronic interstitial inflammation, manifested by connective tissue proliferation and by lymphoid infiltration. In congenital syphilis, because of the widespread localization of the organisms, the diffuse chronic inflammatory portion of the process soon predominates. The primary, essential vascular involvement becomes masked and, finally, so completely hidden as to be recognized with extreme difficulty. When this stage is reached caution is necessary in making the pathological diagnosis, because of the similar appearances produced by the non-specific inflammations discussed earlier. Personally, I would feel much more certain of the correctness of a histological diagnosis of congenital syphilis based upon very slight but characteristic vascular involvement than upon an advanced pneumonia alba.

#### THE NUMERICAL RELATIONSHIP OF *Treponema pallidum* TO NEONATAL SYPHILIS.

As has been stated earlier in this paper the routine examination of all fetal and infantile material for *Treponema pallidum* brought out two facts of considerable importance. Of these, the first, the occurrence of non-specific chronic inflammatory lesions, not to be distinguished histologically from those of undoubted syphilitic origin, has already been discussed. The second, the presence of organisms in cases which show none of the changes characteristic for lues, remains to be considered. Because of the presence of *Treponema pallidum*



in cases which show little or no pathological change it becomes possible to group certain of the cases of hereditary lues. It is to be borne in mind, however, that reference is made only to neonatal syphilis, that form of the disease in which the infection is manifested at birth or shortly thereafter.

The cases with which I have worked fall easily into two groups. The first group includes those cases which show undoubted lesions of a chronic inflammatory nature. Because of the nature of the pathological change we can designate this group "chronic."

The second group shows none of the histological changes characteristic of lues, and I do not think that a histological diagnosis of syphilis is possible or justifiable in these cases. This group, because of the slight degree, or the entire absence of characteristic pathological change, we can designate "acute." I wish it to be clearly understood that the terms "chronic" and "acute" are used only for the sake of convenience to refer to the pathological types of the disease, and that they have no reference to the possible clinical duration of the disease.

To each group belong still-born infants, as well as those born alive. I realize that I am upon dangerous ground when it is attempted to decide that a macerated, still-born fetus does not show the chronic inflammatory evidences of lues. Certain cases, born dead and showing more or less well-marked maceration, may give evidence of chronic inflammation by connective tissue proliferation, lymphoid infiltration, and the other changes associated with neonatal syphilis. In these the proliferating stroma and the infiltrating lymphocytes stain sufficiently well to be recognizable, even when the parenchyma, particularly that of the liver, has practically entirely disappeared. The occurrence of encapsulated gummata in macerated still-born fetuses is not so extremely rare. Other cases show no inflammatory changes, but only the maceration produced by autolysis. It may be argued that the inflammatory tissue in such cases has also undergone autolysis. I have not, however, been able to convince myself that this has occurred in the so-called acute, still-born cases that I have studied. In chronic cases showing much more advanced and complete maceration than was present in any of those that I have placed in the acute group, the inflammatory tissue is still to be made out,

whereas this is not true of the latter group. Mere macroscopic enlargement of the macerated liver, spleen, or other of the internal organs of a still-born fetus is not sufficient basis for a diagnosis of hereditary syphilis. Such enlargement is due to absorption of fluid. It is a part of the process of maceration, and may be just as marked in cases which are certainly not luetic as in those in which the disease is present.

In the chronic group *Treponema pallidum* bears a close relationship, as regards its localization, to the lesions. The organisms are most numerous at the advancing margins of inflammatory zones, and are least numerous in older lesions in which necrosis has begun. Their number varies, of course, with the individual case, but it is never so great as to render impossible the enumeration of organisms present within any given oil immersion field. In the acute group, on the other hand, the parasites may be exceedingly numerous. They may be distributed throughout the various organs of the body, and in any oil immersion field, selected almost at random, their number may be so great that enumeration is impossible.

In the grouping which follows, the chronic group includes only such cases which show undoubted evidences of chronic inflammation. The acute group includes cases which show absolutely no change of this kind whatever or, at most, a very slight and early involvement of the blood vessels of one or more of the organs.

#### A. CASES SHOWING CHRONIC LESIONS.

1. *Born living*.—Of the chronic cases born alive Fig. 1 shows the small number of organisms present in any single field of a well-marked pneumonia alba.<sup>1</sup> The septa are greatly thickened and in the larger portion of the lung the air spaces are completely obliterated. It is of interest to compare Fig. 1 with Fig. 2, both being derived from the same case. Fig. 2 is from the wall of a branch of the portal vein within the liver. The vessel wall shows a diffuse lymphoid infiltration so intense that it is almost impossible to detect any of the original tissue elements. There is very little connective tissue proliferation. Judged by the standards of pathological histology this lesion is not of so long duration as the lung change. From Fig. 2 it will be seen that the number of organisms is considerably greater in the more recent lesion than in the older one.

<sup>1</sup> It will be noticed that there is some variation in the thickness of the organisms, even in photographs made with the same magnification. This is due to differences in the readiness with which the various tissues are penetrated by the impregnating fluids. The possibility of the presence of spiral organisms other than *Treponema pallidum* has been excluded in every case by the use of the Giemsa method upon smears made from the organs at the time the autopsies were performed.

Chronic syphilitic inflammation of the spleen usually results in a considerable relative decrease in the number of lymphocytes and their replacement by the cells and fibers of the stroma. The proliferation is, as a rule, so diffuse that it is impossible



FIG. 1.—Well-marked pneumonia alba.  $\times 1,500$ .

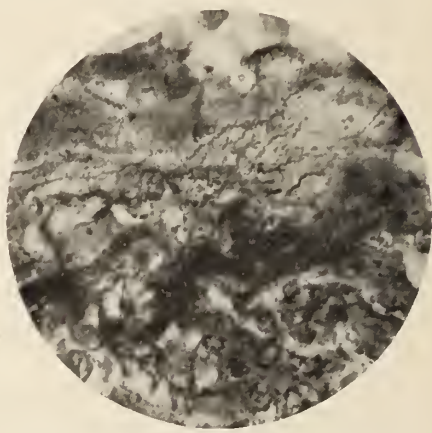


FIG. 2.—From a branch of the portal vein which shows intense lymphocytic infiltration.  $\times 1,500$ .

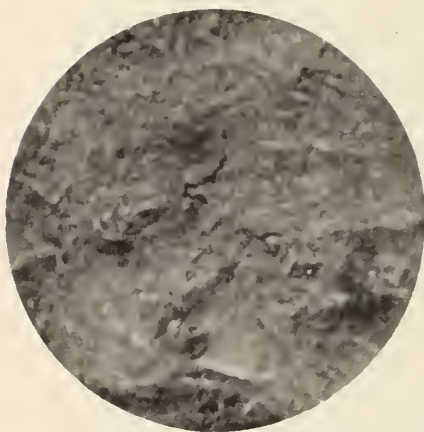


FIG. 3.—From a chronic lienitis which shows disappearance of lymphoid cells and diffuse increase of stroma.  $\times 1,500$ .

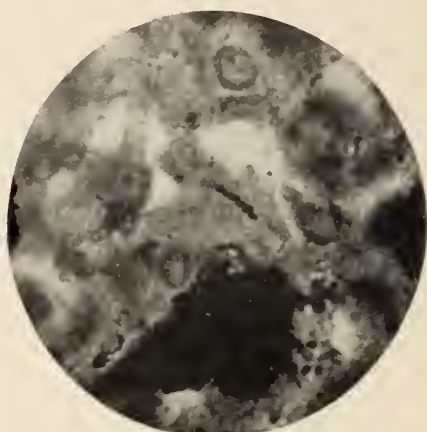


FIG. 4.—A very early miliary pericapillary gumma of the liver.  $\times 1,500$ . The black mass is a necrotic liver cell.

to determine whether the blood vessels show characteristic involvement. Fig. 3, from such a spleen, shows only a single treponema within the field.

In one case included in the chronic group, an infant which died three weeks after birth, the liver shows miliary gummata of an earlier stage than I have ever seen in acquired syphilis. The lesions bear a very definite relationship to the capillaries,



either entirely surrounding a capillary or being situated at one side of it. They are composed of fibroblasts, polymorphonuclear eosinophiles, and lymphocytes intermingled with each other. Necrosis is absent or is limited to individual liver cells.

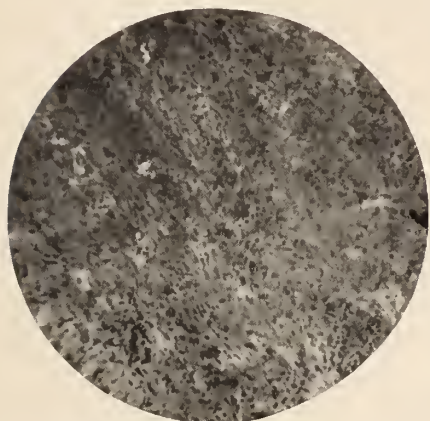


FIG. 5.—Portion of the fibrous capsule of a necrotic gumma of the lung.  $\times 100$ .

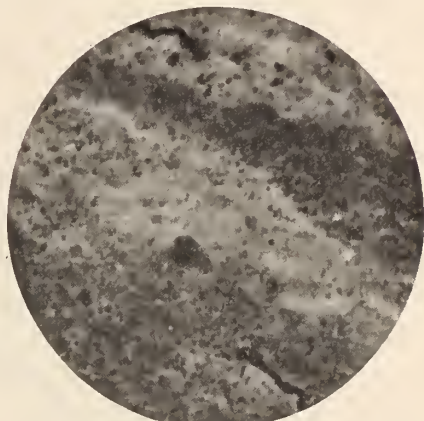


FIG. 6.—From the tissue shown in Fig. 5.  $\times 1,500$ .

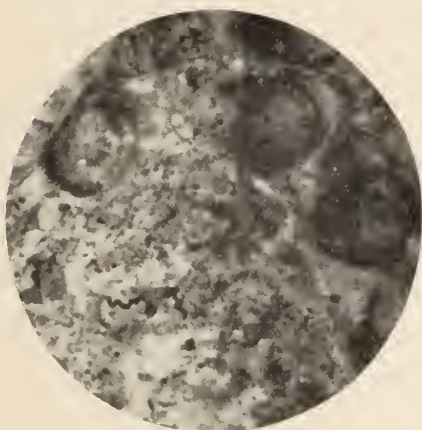


FIG. 7.—From a thickened and infiltrated alveolar septum of the lung.  $\times 1,500$ .

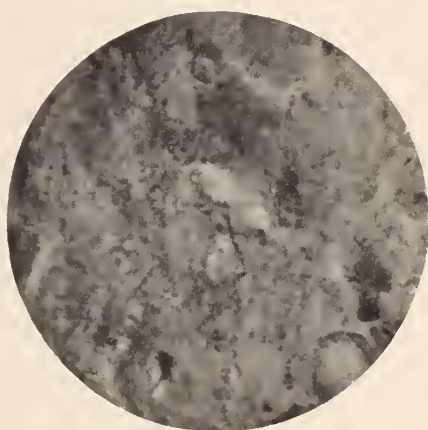


FIG. 8.—From a spleen which shows few lymphocytes and increased prominence of the stroma nuclei.  $\times 1,500$ .

The centers of the gummata, even at such an early stage, show very few organisms, as appears from Fig. 4. At the periphery, within liver cells, they may be somewhat more numerous.

2. *Still-born*.—The still-born chronic group offers interesting preparations, more particularly when a comparison is made with those derived from the still-born acute

group. Fig. 5 is the thick, fibrous, infiltrated capsule of a necrotic gumma of the lung. Fig. 6, from a portion of the capsule, shows two organisms. They are thick, the spirals are irregular and in part lost. As the result of many investigations it is well known that *Treponema pallidum* decreases rapidly in numbers in the skin lesions of the late secondary stage of the acquired disease, that is, when necrosis

begins. It is present in exceeding paucity in the necrotic gummata of acquired lues. This disappearance of the organisms with the beginning of necrosis may be partly due to an active migration away from the lesion. Fig. 6 indicates that the decrease is in part, possibly in large part, due to degeneration and death of the organisms themselves, caused by the same processes which induce the tissue death. Fig. 7 shows two organisms in a field from an alveolar septum of the lung. Lymphocytes are numerous and the septum is thicker than normal.

Fig. 8 is from a spleen in which maceration has proceeded to such an extent that only the nuclei of the lymphocytes and of the fibrous stroma stain. The stroma is prominent.

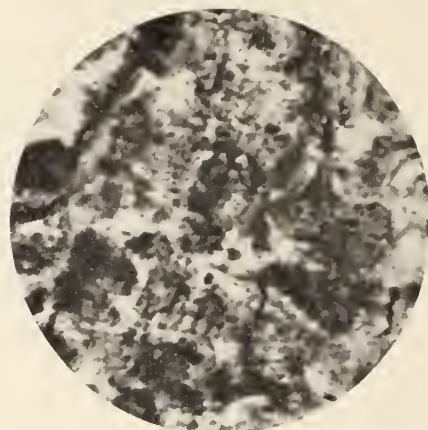


FIG. 9.—From the periglomerular connective tissue of the kidney. The interstitial tissue is greatly increased in amount and is rich in young spindle nuclei and in lymphocytes.  $\times 1,500$ .

Fig. 9 illustrates the small number of organisms present in the periglomerular connective tissue of the kidney. The organs of the fetus from which the preparation was derived were so macerated that the parenchyma cells are not stainable. In the kidney the tubules are recognizable only as unstained, granular areas. The glomerular nuclei still stain well. Newly formed connective tissue infiltrated with lymphocytes widely separates the glomeruli and the tubules.

#### B. CASES SHOWING NO CHARACTERISTIC LESIONS.

1. *Born living*.—My material illustrating the acute, born-living group is limited to one case, an infant which died a few days after birth. For two or three weeks previous to her confinement the mother had complained of a loss of hair and of sore throat. Six weeks after the birth of the child she developed a typical skin eruption. The lungs of the infant show an acute pneumonia of lobular type, but absolutely none of the histological changes which might warrant a diagnosis of lues. Fig. 10 gives some idea of the richness of the pulmonary tissue in organisms. The spleen shows only acute congestion, but many treponemata. The adrenal, Fig. 11, is also rich in organisms. The only organ which shows any histological change that may be considered specific is the liver. Some of the vessels are surrounded by a narrow zone in which there is a very moderate grade of lymphoid infiltration. In comparing Fig. 1 with Fig. 2, both from a chronic case, the statement was made that treponemata are con-

siderably more numerous in the liver, the organ in which the lesions are, pathologically speaking, more acute than in the lung, the seat of more chronic changes. The same is true for the acute case under consideration. Whereas organisms are numerous in

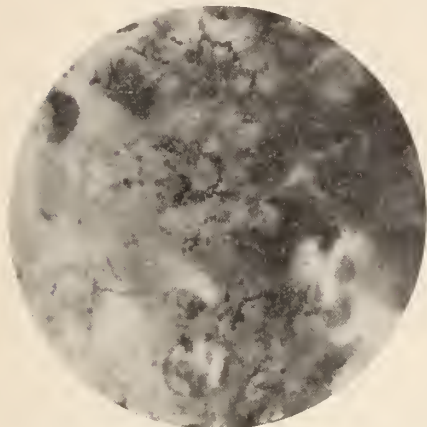


FIG. 10.—From the bronchial cartilage of a lung which shows no chronic change.  $\times 1,800$ .

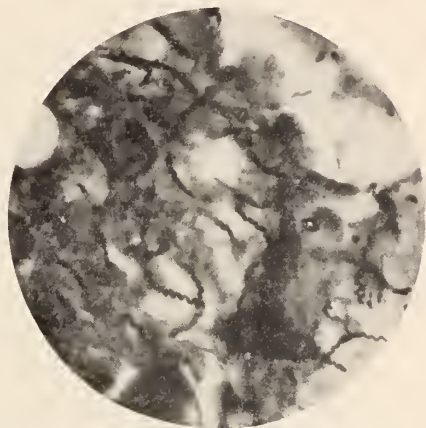


FIG. 11.—From an adrenal which shows only congestion of the medulla.  $\times 1,800$ .

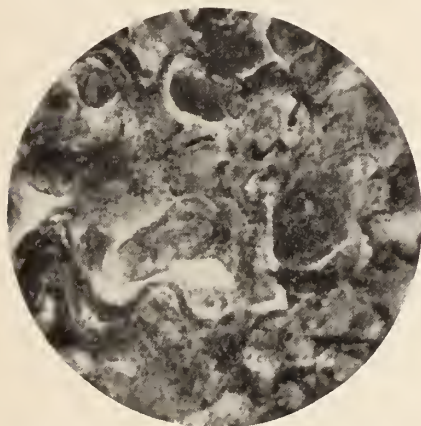


FIG. 12.—The wall of a branch of the portal vein.  $\times 1,500$ .

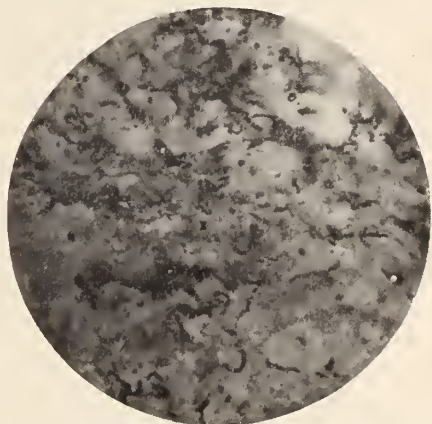


FIG. 13.—From a lung which shows maceration but no chronic change.  $\times 1,500$ .

those organs which show no specific change, they are present in small numbers in the liver (Fig. 12), the only organ which shows any characteristic lesion.

2. *Still-born*.—The remaining photographs were made from the tissues of fetuses, none of whose organs show any histological inflammatory changes. Fig. 13 is from an alveolar septum of the lung of a fetus of approximately the same age as that from which



Fig. 7 is derived. Maceration was about equally marked in the two cases. The septum illustrated in Fig. 13 is of normal thickness and shows no increase in lymphoid cells or in fibroblasts.

Fig. 14 is to be compared with Figs. 3 and 8. In the spleen of Fig. 14 the nuclei of the small lymphoid cells and of the connective tissue cells still stain fairly well. The stroma is not unduly prominent. A comparison of Fig. 14 with Figs. 3 and 8 may help in establishing the chief point under discussion, the numerical relationship of *Treponema pallidum* to two well-characterized groups of neonatal syphilis.

Furthermore, compare Fig. 15 with Fig. 9. The kidney from which the former is derived shows no periglomerular or interstitial change, yet organisms are numerous. None of the organs of the fetus from which the kidney of Fig. 15 comes shows any greater inflammatory change than does the kidney. *Treponema*

FIG. 14.—Spleen. Although maceration is present the stroma nuclei stain and are not increased in number.

are as numerous in all the other organs as they are in the kidney. In the preparation of the photomicrographs the attempt was made to select representative fields, fields which would make possible a just comparison between the cases showing chronic inflammatory changes and those showing no specific change. The preponderating number of organisms in the latter group is even more striking when the sections themselves are studied than appears from the photographs. It is well to state explicitly that I do not wish to maintain that every case of neonatal syphilis which shows well-marked chronic lesions will be characterized by a paucity of organisms. Although all of the chronic cases studied by me do show small num-

bers of *treponemata*, as compared with those cases which show no chronic lesions, there is no reason why a chronic inflammatory process might not be associated with a large number of parasites. To properly explain such a case, however, it would be necessary to suppose a moderate infection leading to tissue reaction, followed by a subsequent rapid multiplication of organisms. Such a condition is exactly comparable

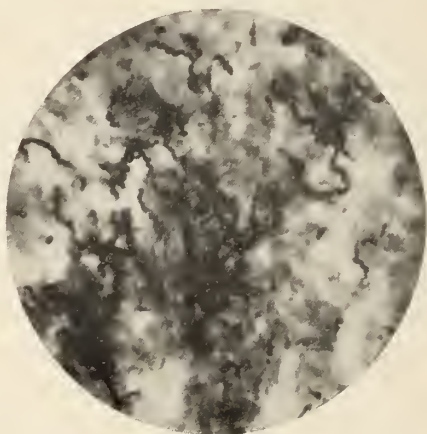


FIG. 15.—From the peri-arterial tissue of the kidney. The interstitial tissue of the organ is not increased.

with a bacteriemia following a chronic abscess, an acute generalized miliary tuberculosis following a chronic ulcerative tuberculosis of the lung, or a fatal paroxysm occurring in the course of a chronic estivo-autumnal malaria.

#### EXPLANATION OF THE VARYING NUMERICAL RELATIONSHIP.

Is it possible to satisfactorily explain the varying numerical relationship of *Treponema pallidum* to those cases, on the one hand, which show characteristic tissue changes, and, on the other, to those which show no chronic lesions? Essentially, the question seems to be one of the grade of infection, although the factor of individual resistance cannot be entirely eliminated. An absolutely exact answer cannot be given, because the parental history in congenital lues is so untrustworthy. The exact period, during the intra-uterine life of the fetus, at which infection occurs cannot be accurately estimated. It seems reasonable to suppose that in the chronic cases the infection is one of only moderate severity, one which permits a reaction on the part of the tissues. In a certain percentage of such cases the development of the lesions proceeds at such a rate that the life of the fetus is finally destroyed in utero during the late months of pregnancy. In other cases, because of a still milder infection and a slower degree of development of pathological change, a living infant is born. The post-mortem findings, whether the child is still-born or is born living, are those of the classical picture of hereditary lues.

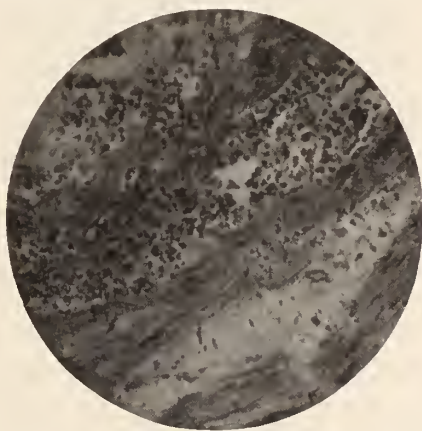


FIG. 16.—Outer portion of the wall of the umbilical vein. Separation of the muscle bundles and marked infiltration by lymphocytes.  $\times 150$ .

In cases which show no chronic lesions, the ones in which organisms are so numerous, it would seem that one is dealing with an infection of extreme severity from its very inception. There results an overwhelming of the body of the fetus, causing death before there is time for the tissues to react sufficiently to lead to the development of chronic

inflammatory changes. This view may appear more or less speculative. But so long as the exact moment of fetal infection cannot be determined, even speculation may help in arriving at probabilities. That there is some basis of fact in the idea expressed appears from a detailed study of the case from which Fig. 13 is derived. All of the organs of the fetus show an astonishing richness in organisms without any inflammatory changes. The outer zone of the wall of the umbilical vein (Fig. 16) shows a marked lymphoid infiltration.



FIG. 17.—From the umbilical vein shown in Fig. 16.  
× 1,500.

In these areas of pathological change *treponemata* are present (Fig. 17), but only in moderate numbers. An occasional large chorionic vein shows the same change in somewhat less marked degree and with fewer organisms. A study of the histopathology of this case leads to the belief that the primary localization of the parasites occurred in the walls of the umbilical and chorionic veins,

and that from these foci of infection the fetus was invaded. The invasion resulted in an "Ueberschwemmung" of the fetus and in the production of death before there was time for the establishment of fetal inflammatory lesions like those present in the cord.

#### THE CAUSES OF DEATH IN CONGENITAL LUES.

In the chronic group the lesions present offer a sufficient explanation of the cause of death. Very probably a chronic toxemia also plays some part in the death of the fetus. In the acute group death is due to the large number of organisms present, just as a streptococcus bacteriemia may cause death without the production of inflammatory lesions. Besides the cases in which fetal death is due directly to *Treponema pallidum*—either by the production of pathological changes or because of its presence in very great numbers—it is possible that syphilis on the part of the mother may cause the death of

the fetus without the intervention of either of these factors. In such cases death must be due to the transmission of toxic substances from the mother to the fetus or to disturbances of fetal metabolism, and is not due directly to syphilitic involvement of the fetus. Some such mechanism as this must be the cause of fetal death in those cases in which the mother gives undoubted evidence of lues, but in which the fetus shows no chronic inflammatory changes and no treponemata.

#### GERMINAL INFECTION.

One is forced to consider, for a space, the possibility of germinal infection in syphilis. As our knowledge of the etiology of the infectious diseases has increased, the possibility of an infection of a germinal cell followed by the development of a specifically diseased fetus has become more and more limited. At the present time such an infection receives serious consideration only when one is dealing with tuberculosis and with syphilis. When it is borne in mind that the most probable immediate result of an invasion by the tubercle bacillus is an intra-cellular infection, which leads primarily to hypertrophy of the invaded cell and to a disturbance of its normal regulatory mechanism—a process resulting in the formation of a giant cell—the possibility of infection of an individual spermatozoon or ovum without a destruction of the viability of the cell appears extremely remote. It is true that a number of the pathogenic protozoa may invade the ovum without interfering with its further development. More particularly, the germinal infection of the secondary insect host is a phenomenon which distinguishes certain of the protozoa very sharply from the great majority of the pathogenic bacteria. While the semen in syphilis may be infectious, a parasitization of individual spermatozoa appears extremely improbable. The presence of *Treponema pallidum* within the ova of infants infected with congenital lues has been reported. I am able to confirm the correctness of this observation in one of my own cases. That an infection of an ovum of a sexually mature woman is a physical possibility must, therefore, be admitted. There is, however, ground for a difference of opinion as to the ultimate result of such an infection upon the development of the ovum after fertilization. It may be possible that the very early abortions of syphilis are the results of the infection of

ova. That the development of such embryos could proceed very far does not seem probable, when one recalls that the presence of *Treponema pallidum* causes either a marked inflammatory reaction on the part of the tissues when the infection is not too severe, or death before lesions can be produced, if large numbers of organisms are concerned in the invasion. I am unwilling to admit the probability, or even the possibility, of germinal infection in those cases which constitute the condition known as congenital syphilis—infants born dead during the latter half of pregnancy and those born living at or shortly before the end of gestation and manifesting at birth or shortly thereafter the evidences of lues.

#### SUMMARY.

The presence of *Treponema pallidum* is the only accurate criterion for the diagnosis of congenital lues.

In infants, both still-born and born living, there occur, relatively frequently, chronic inflammatory changes which render the histological diagnosis of syphilis extremely difficult.

The essential lesion, in congenital as well as in acquired lues, is the vascular involvement. This begins with the localization of the parasites in the perivascular lymphatics, which leads first to proliferation and infiltration of the adventitia and then to a spread of the inflammatory process inward toward the lumen of the vessel as well as outward into the tissues surrounding the vessel. In the congenital form of the disease the diffuseness of the interstitial inflammation may overshadow and hide the more essential and characteristic vascular involvement.

The routine examination of all infant autopsies for *Treponema pallidum* makes possible a classification of neonatal syphilis into two groups.

In the first group belong those cases showing vascular involvement and chronic interstitial inflammatory changes.

In the second group belong cases which show none of the lesions that can be considered characteristic of lues.

In the first group organisms are, as a rule, not numerous, and death is due in part to the pathological change and in part to toxemia.

In the second group organisms are present in astonishing numbers, and death is due to an overwhelming of the fetus. Death results,



possibly through acute intoxication, before there is sufficient time for a reaction on the part of the tissues.

To each group belong still-born fetuses, as well as infants born living.

That the cases of congenital syphilis born during the latter half of pregnancy are the results of germinal infections appears improbable and impossible.

# THE RELATION OF THE OPSONIC INDEX TO THE LEUCOPENIA AND LEUCOCYTOSIS FOLLOWING INJECTIONS OF HEATED BACTERIAL CULTURES.\*†

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(From the Laboratory of Pathology and Bacteriology of the Health Department, St. Louis, Mo.)

IN the course of a series of experiments on rabbits on the effect of injections of killed bacteria on the leucocytes of the circulating blood, it was found, as pointed out by others, that an initial leucopenia was produced which was followed by a marked leucocytosis. It occurred to us that possibly some relation existed between the leucopenia and the negative phase, and the leucocytosis and the positive phase, of the opsonic index. If such a relation should prove constant, it might be of some practical as well as theoretical significance. There seemed to be a slight possibility of substituting simple leucocyte counts for the cumbersome opsonic technique as a means of determining the reaction of the individual to the injection of dead bacteria, and with this idea in mind our animal experiments were continued.

Metchnikoff<sup>1</sup> and his pupils found that intraperitoneal and intravenous injections of a culture of cholera vibrios were followed by an immediate diminution in the number of the leucocytes. Howard<sup>2</sup> showed: (1) that intraperitoneal injections of cold salt solution into guinea-pigs caused a slight leucocytosis in four or five hours; (2) that intraperitoneal and subcutaneous injections of typhoid toxins and intraocular injections of killed typhoid bacilli produced a very rapidly developing and marked primary hypoleucocytosis which was followed by a marked hyperleucocytosis.

Staubli<sup>3</sup> made intraperitoneal injections of typhoid toxin and noted a primary hypoleucocytosis followed by a considerable hyperleucocytosis. Dean<sup>4</sup> found that after injection of diphtheria toxin

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<sup>1</sup> Kolle and Wasserman, *Handbuch*, 4, p. 380.

<sup>2</sup> *Jour. Med. Res.*, 1907, 17, p. 237.

<sup>3</sup> *Deut. Arch. f. klin. Med.*, 1905, 85, p. 286.

<sup>4</sup> *Jour. Path. and Bact.*, 1908, 12, p. 154.

there was an immediate and continuous rise in the number of the leucocytes of the circulating blood except when a neutralizing dose of antitoxin was given at the time of, or shortly before, the injection of the toxin. Marshall<sup>1</sup> found that intravenous injection of large doses of killed staphylococci caused a slight primary rise, followed in 25 hours by a fall, in the opsonic index; but by dilution experiments the phagocytosis was found to fall quickly to a minimum.

#### TECHNIQUE OF THESE EXPERIMENTS.

In these experiments healthy medium-sized rabbits were used. The injected material consisted of from one to six 24-hour agar slants of *B. coli* or *B. pyocyaneus* suspended in physiological salt solution and heated to 70° C. for two hours. Fifteen rabbits were used. Eight were injected with *B. pyocyaneus*, six with *B. coli*, and one with cold salt solution. Blood counts were made and blood collected for the opsonic determination immediately before, and at intervals of 20 minutes to four hours after, the injection for the first day, and once or twice daily thereafter. The usual care was observed in collecting the blood.

We are inclined to agree with Bunting<sup>2</sup> that rabbits are satisfactory animals for experimental blood work. He found that "counts made on successive days have shown only the slightest variations, either in the total count or in the differential." In our experiments an average of 18 counts on normal rabbits, taken without reference to time of day or feeding, gave 8,500 leucocytes per cu. mm. By far the greatest number ranged between 6,000 and 9,000.

The usual technique was employed in estimating the opsonic content of the serum. In the case of *B. pyocyaneus*, however, a special difficulty was encountered. After incubating a mixture of the serum, leucocytes, and suspension of these bacteria, it was almost impossible to find an intact leucocyte in the stained smear. The cytoplasm showed marked "digestion," and in certain instances only the nuclei, markedly disintegrated and indistinctly stained, could be found.

In 1899, Gheorghiewsky<sup>3</sup> noted that in 15 to 20 minutes after

<sup>1</sup> *Jour. Path. and Bact.*, 1908, 12, p. 378.

<sup>2</sup> *Jour. Exp. Med.*, 1908, 8, p. 620.

<sup>3</sup> *Ann. de l'Inst. Pasteur*, 1880, 13, p. 298.

intraperitoneal injection of suspensions of *B. pyocyaneus*, the leucocytes obtained from the peritoneal cavity had lost their motility, had become round and swollen, and showed abnormal staining of their chromatin. *In vitro*, he found degeneration of the leucocytes when mixed with a suspension of *B. pyocyaneus*. In order to prevent this "digestive" action on the leucocytes in our mixtures it was necessary to suspend the organisms in physiological salt solution, centrifuge them for an hour, pipette off supernatant fluid, resuspend them in salt solution, and again centrifuge them for a few minutes to get rid of clumps. Normal rabbit serum contains a strong agglutinin for *B. pyocyaneus* which also interferes with opsonic technique. This difficulty could be obviated only by diluting the sera.

#### RESULTS.

Of the fifteen rabbits used, five died. Two of these had been given *B. coli* and three *B. pyocyaneus*. Four died within five hours after the injection, the fifth died on the second day. The four dying quickly showed practically the same condition; that is, a very prompt and pronounced fall in both leucocytes and opsonins. Forty-five minutes after intraperitoneal injection of three 24-hour glycerine agar slants of *B. pyocyaneus* which had been heated to 66° C. for two hours, the number of leucocytes had dropped from 5,800 to 1,100 per cu. mm. At the same time, the phagocytic index fell from 2.3 to 0.15. There was a very slight fluctuation about these low figures until death occurred in convulsions four hours after injection (Chart 1). In the case of a rabbit injected with four 14-hour glycerine agar slants of *B. coli* heated to 66° C. for two hours, in one hour and 20 minutes there was a fall in leucocytes from 7,600 to 1,800 and in the opsonic index from 0.9 to 0.25. Death occurred in convulsions two hours and 25 minutes after injection without any noteworthy change in the leucocytes or opsonins (Chart 2).

The animal which lived until the second day differed somewhat in its reaction. It received four 12-hour glycerine agar slants of *B. pyocyaneus* killed at 66° C. There was a prompt fall in the number of leucocytes from 4,000 to 800, after which the number increased gradually. The opsonic index at the same time rose from 0.6 to 1.1 and remained at about this point for the remainder of the day.

Shortly before death the leucocytes had risen to 3,000 and the opsonic index had fallen to 0.5.

In the case of the rabbits which survived, whether subcutaneous or intraperitoneal injections were given or either organism used, the results were practically identical. In every case there was a prompt fall in the number of the leucocytes. The lowest point was reached from three to seven hours after injection. The diminution in number varied from 2,000 to 7,700 leucocytes per cu. mm., the average

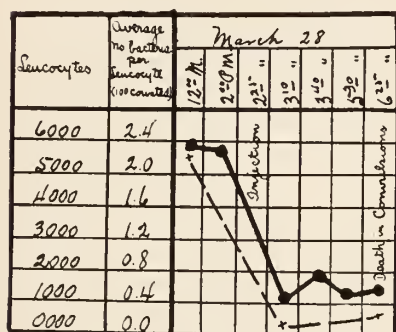


CHART 1.—Solid line=Leucocytes. Broken line=Opsonic Index. Intraperitoneal injection of three agar slants. *B. pyocyaneus*. Killed at 66° C.

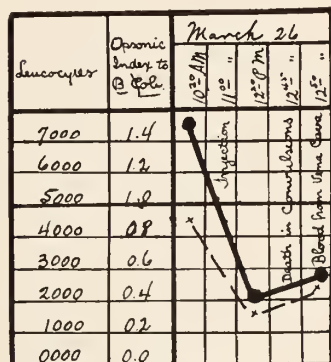


CHART 2.—Solid line=Leucocytes. Broken line=Opsonic Index. Intraperitoneal injection of four 14-hour agar slants. *B. coli*. Killed at 66° C.

fall being 4,800. On reinoculation the lowest point was reached in from one-half to two hours.

During the first two to eight hours after injection the opsonic index showed little variation; in some instances there was even a slight rise. After this a rather gradual decline took place, the lowest point being reached on the second to the fourth day. The negative phase of the opsonic index is thus seen to really begin about the time, or very shortly before, the period of leucopenia ends (Charts 3, 4, and 5).

On the second to the fourth days, usually the second and third, after injection, the leucocytes reached their highest point. In one case the count reached 37,000 per cu. mm. The increase in the number of leucocytes varied from 1,500 to 31,000 per cu. mm. above the count made previous to inoculation. On the third or fourth day the leucocytes dropped rather suddenly to normal and remained

practically within normal limits. In a few instances (Chart 3) a secondary leucopenia occurred, as mentioned by Howard.

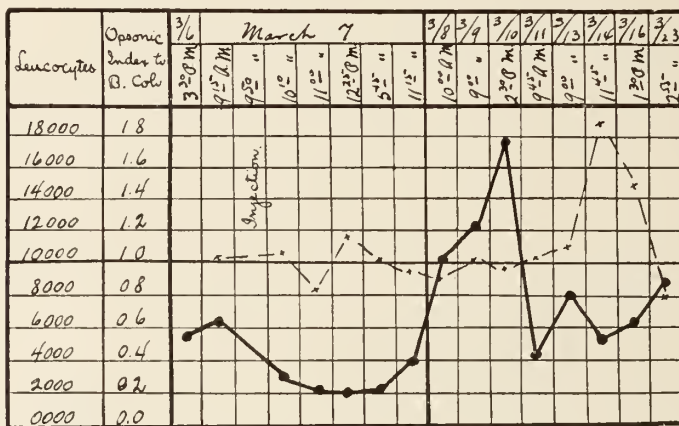


CHART 3.—Solid line=Leucocytes. Broken line=Opsonic Index. Intraperitoneal injection of three agar slants, *B. coli*. Killed at 66° C.

The opsonic index rose more gradually and reached its maximum on the fourth to the eighth days after the first injection, and on the

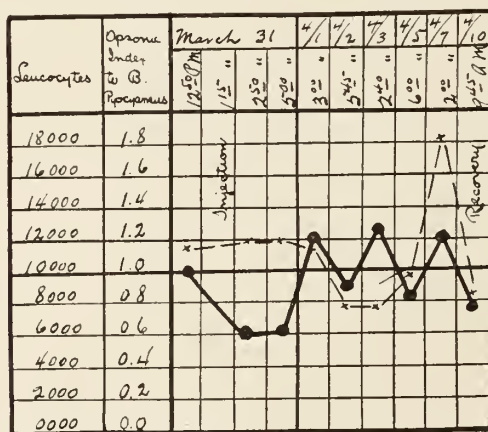


CHART 4.—Solid line=Leucocytes. Broken line=Opsonic Index. Subcutaneous injection of three agar slants, *B. pyocyaneus*. Killed at 66° C.

fourth to the tenth days after the second injection. An average of all the estimates made showed the highest indices on the sixth day. In the course of 10 to 12 days the opsonic index returned to normal



and sometimes remained below normal for two or three days. Thus it is seen that the positive phase of the opsonic index bears practically the same relation to the period of leucocytosis that the negative phase bears to that of leucopenia. The opsonic reaction to the injection is of slower development and of longer duration than the leucocytic reaction. Not infrequently the period of greatest leucocytosis was coincident with that of the lowest opsonic indices.

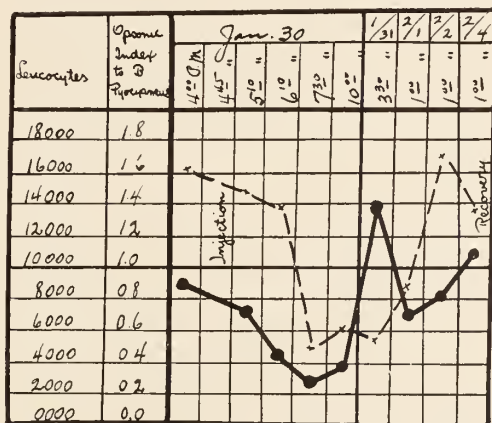


CHART 5.—Solid line=Leucocytes. Broken line=Opsonic Index. Intraperitoneal injection of four agar slants. *B. pyocyaneus*. Killed at 66° C.

Special attention may be called to two practical deductions from these experiments. First, the conditions found in the animals that died suddenly suggest the possibility that in so-called foudroyant infections in man, the sudden overwhelming of the human organism is rendered possible only by a complete paralysis of its entire protective mechanism. In all four of these animals both leucocytes and opsonins fell to a most abnormally low point and showed no signs of reacting. It does not seem unreasonable to suppose that the lysins and other protective bodies in the serum underwent similar changes. In all the animals that recovered, some of which received larger doses than the ones that died, the opsonins held their own until the leucocytes had begun to increase in number. This idea is further borne out by the results of injection of live bacteria (see Chart 6). When both leucocytes and opsonins fell quickly and showed no tendency to react,



the animal died quickly; but when the opsonins showed little or no change until the number of leucocytes began to increase, the animal usually recovered, or at least lived several days.

Second, intraperitoneal injections have a more powerful action on the leucocytes than do subcutaneous injections. (Compare Charts 3 and 4.) This is probably due to the more rapid absorption

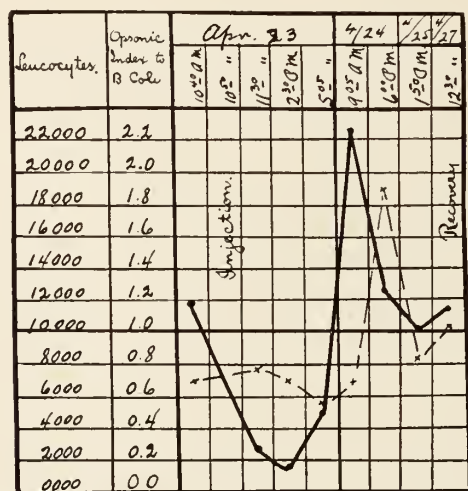


CHART 6.—Solid line=Leucocytes. Broken line=Opsonic Index. Intraperitoneal injection of one agar slant of living *B. coli*.

of the injected material from the peritoneal cavity. On the other hand, as already pointed out by one of us,<sup>1</sup> subcutaneous injections have an equally, if not more, favorable action in stimulating the production of opsonins.

#### SUMMARY.

Briefly summarized, then, the injection of heated bacterial cultures causes, in from 20 minutes to four hours, a leucopenia, followed, in two or three days, by a marked leucocytosis. The leucocytes usually return to the normal in four days. The opsonic index shows little or no variation for the first few hours after injections. The negative phase then sets in and lasts from two to four days. After this there

<sup>1</sup> *Jour. Infect. Dis.*, 1907, 4, p. 595.

is a gradual rise, the maximum being reached on the fourth to the eighth days. A gradual fall to normal occurs between the sixth and twelfth days. The leucocytes, therefore, are evidently more sensitive to bacterial injections than the opsonic index. Their variations are greater, the period of greatest leucopenia precedes the greatest fall in the opsonins, and the period of greatest leucocytosis precedes the greatest rise in the opsonic index, which usually reaches its maximum only after the leucocytes have returned to normal.

## THE ORAL ADMINISTRATION OF ANTITOXINS.\*

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THIS paper is a report of the later results of a series of investigations which were published in this *Journal*<sup>1</sup> in 1906. At that time our results warranted us in concluding that diphtheria and tetanus antitoxins, when given per mouth to experimental animals, were absorbed in sufficient quantities to protect those animals against several fatal doses of diphtheria and tetanus toxins. We were also able to show that the oral administration of antidiphtheric and anti-tetanic serums to children was followed by an absorption of the antitoxins, particularly that of diphtheria, in sufficient quantities to show markedly antitoxic properties in the blood of the treated individuals.

These results were obtained by the use of certain drugs which apparently inhibited digestion and promoted absorption. There was also reported some preliminary work showing that the toxins of diphtheria and tetanus were absorbed from the alimentary canal of guinea-pigs, provided that digestion was inhibited.

A series of five children were injected subcutaneously with from 320 to 1,040 units of diphtheria antitoxin, and careful tests showed that only a part of this could be detected in the blood of the treated individuals. A more direct comparison between the oral administration and the hypodermic injection could not be drawn. However, assuming that the antitoxin should be given to children as a prophylactic, we believed that the results obtained from the administration per mouth compared very favorably with those from subcutaneous injection.

After having carried on the work far enough to show that toxins and antitoxins, under certain conditions, are absorbed with a reasonable degree of uniformity when administered per mouth, we attempted to demonstrate more precisely the conditions necessary for such absorption. In our previous work it was found that the most uniform results were obtained when trikresol, salol, chloroform, and opium were

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added to the antitoxins before administration per stomach. The question remained whether these were the best drugs to use and in what quantities they should be given. It also appeared to us that the absorption of diphtheria toxin, when administered per mouth, should be determined for the horse, to confirm and strengthen previous results and also to suggest a possible practical application of the oral method for immunization.

In our former report no definite conclusion could be drawn in regard to the rate of absorption of the antitoxins and toxins from the alimentary canal. This phase of the problem seems to us to be of considerable clinical importance.

It was also necessary to know from actual experiment whether dry serum could be given orally and absorbed with the same degree of uniformity as the liquid antitoxin. This we believe to be of practical consequence, if the oral method should ever be used as a prophylactic in children.

Finally we decided to conduct a series of experiments on children, treating some by hypodermic injection and others by oral administration of antitoxin and, by testing the blood of the children so treated for its antitoxic content, to be able to make a direct comparison between the two methods of treatment.

#### EXPERIMENTS RELATIVE TO THE NATURE AND QUANTITY OF DRUGS BEST ADAPTED TO THE PROMOTION OF ABSORPTION AND INHIBITION OF DIGESTION WHEN GIVEN ORALLY WITH ANTITOXIN OR TOXIN.

A series of 64 guinea-pigs were treated with tetanus toxin per stomach, and on this series various drugs in different combinations were tried. Likewise, a series of 12 guinea-pigs and seven rabbits, used for the same purpose, were treated per mouth with antitoxin. Some of the drugs which were tested on the above animals were atropine, codeine, hyoscyanine, morphine, cocaine, and sodium bicarbonate. These were tried to find out whether any one or several in combination would give better results than the opium, salol, and chloroform. Experiments were also conducted to ascertain whether opium, salol, or chloroform could be omitted.

The following series of 29 guinea-pigs were treated with dry

antitoxic serum, to which were added only opium and salol, the chloroform being eliminated. Twenty-seven of these animals were treated per mouth, each with 1 gram. of dry diphtheria antitoxin, to which was added 0.05 gram. of opium and 0.1 gram. of salol. Before treatment they received 5 c.c. of a 1 per cent solution of sodium bicarbonate. The series of pigs were then injected subcutaneously with 3.5 fatal doses of diphtheria toxin. The two control and four of the treated pigs died in two days. At the end of four days 17 of the treated pigs had died, and the remaining 10 out of 27 remained well. In the above experiment the series of pigs received a rather heavy dose of toxin, and the fact that 10 of the pigs withstood  $3\frac{1}{2}$  fatal doses, shows considerable absorption of the antitoxin without the use of the chloroform.

In the next experiment sodium bicarbonate was given about one-half hour before the administration of the antitoxin, and much better results were obtained.

Table 1 gives the details of this series of experiments. A study of the table will show clearly that the administration of a sodium bicarbonate solution one-half hour before the toxin promotes absorp-

TABLE 1.

SODIUM BICARBONATE AS AN AID IN PROMOTING ABSORPTION OF ANTITOXIN.

One per cent sodium bicarbonate given one-half hour before animals were treated per stomach with toxins. Control animals treated with the same amount of toxin, prepared with the same amount of drugs, but sodium bicarbonate not given before treatment.

Animal Used	Material Used in Treating Animals	No. Control Animals. Not Treated with Sodium Bicarb. Before—All Lived	No. Animals Treated with Sodium Bicarb. $\frac{1}{2}$ Hour Before Administration	No. Animals Treated with Sodium Bicarb. Which Died	No. Animals Treated with Sodium Bicarb. Which Lived
Guinea-pigs.....	Tetanus toxin 100 $\times$ f. d.	1	3	1	2*
Guinea-pigs.....	Tetanus toxin 100 $\times$ f. d.	4	2	1	1*
Rabbits.....	Diph. toxin 250 $\times$ f. d.	3	1	1	0
Rabbits.....	Diph. toxin 250 $\times$ f. d.	2†	1	1	0
Rabbits.....	Diph. toxin 500 $\times$ f. d.	2§	1	1	0
Rabbits.....	Diph. toxin 500 $\times$ f. d.	1	1	1	0

\* Had marked symptoms and recovered. None of the pigs which did not receive the sodium bicarbonate showed symptoms.

† One of these rabbits died seven days after receiving the toxin. The rabbits treated with sodium bicarbonate, before administration of toxin, died two days after receiving the toxin.

§ Both of these died seven and eight days after receiving toxin. The rabbit treated with sodium bicarbonate died in two days.



tion probably by neutralizing the hydrochloric acid in the stomach and thus inhibiting digestion.

#### THE ABSORPTION OF TOXINS.

A series of 29 rabbits and 16 guinea-pigs were used in verifying former results and studying the absorption of diphtheria and tetanus toxins. The administration of snake venoms per mouth, with and without drugs, was tried on a series of 17 guinea-pigs. Three horses were treated with diphtheria toxin per stomach. The results of the work on the series of the 29 rabbits are given in Table 2. A study of the table will show that the results are, on the whole, fairly uniform and they confirm our previous observations that diphtheria toxin, under certain conditions, is absorbed from the alimentary canal. Some of the rabbits, given in Table 2, were used in testing different drugs and others in studying the rate of absorption.

TABLE 2.  
DIPHTHERIA TOXIN, PER STOMACH, RABBITS.

Rabbit No.	No. Fatal Doses of Diphtheria Toxin	Drugs Added to Toxin	Result
Control 1.....	300	No drugs	Lived
" 2.....	500	" "	Dead 14 days
" 3.....	250	" "	Lived
" 4.....	None	Broth and drugs, same proportion as treated animals	"
" 5.....	"	Broth and drugs, same proportion as treated animals	"
6.....	300	Usual drugs	Dead 3 days
7.....	300	" " and atropine	" 2 "
8.....	300	" " without opium	" 10 "
9.....	500	" " with "	" 4 "
10.....	500	" " flushed out after 2 hrs.	" 1 "
11.....	500	" " not flushed out	" 1 "
12.....	250	" " Na <sub>2</sub> CO <sub>3</sub> before	" 2 "
13.....	250	" "	" 7 "
14.....	250	" " Na <sub>2</sub> CO <sub>3</sub> before	" 2 "
15.....	500	" " flushed out after 1 hr.	" 3 "
16.....	500	" " Na <sub>2</sub> CO <sub>3</sub> before	" 3 "
17.....	500	" "	Lived, sick 3 days
18.....	500	" "	Lived
19.....	500	" "	"
20.....	250	" " flushed out after 1 hr.	"
21.....	250	" " with sod. bicarb.	"
22.....	250	" " " "	"
23.....	500	" " flushed out after 1 hr.	"
24.....	400	" "	"
25.....	400	" "	"
26.....	200	" "	"
27.....	200	" " with codeine	"
28.....	200	" " " atropine	"
29.....	200	" " " hyoscyanine	"

In the same way 38 guinea-pigs were treated with tetanus toxin and the results were fairly consistent with those already obtained.

A series of guinea-pigs were treated per stomach with rattlesnake and cobra venoms. The results were positive. A few guinea-pig injections, subcutaneous, showed that 0.015 gram. of each venom would kill a 300 gram. pig in one hour. The results of the oral administration are given in Table 3.

TABLE 3.

No. OF PIG	SNAKE VENOMS		PER STOMACH, GUINEA-PIGS	
	Species of Venom	No. Grams of Venom Given	With or With- out Usual Drugs	Results
1.....	Rattlesnake	0.1	Without	No symptoms
2.....	Cobra	0.1	"	"
3.....	Rattlesnake	0.1	With	Dead 30 hours
4.....	Cobra	0.1	"	" 4 "
5.....	Rattlesnake	0.05	"	" 24 "
6.....	Cobra	0.05	"	" 24 "
7.....	Rattlesnake	0.01	"	Symptoms 24 hrs. Recovery
8.....	"	0.01	Without	No symptoms
9.....	Cobra	0.01	With	"
10.....	"	0.01	Without	"
11.....	No venom, drugs alone			"
12.....	" " " "			"
13.....	Control not treated			Healthy

It will be seen, by reference to Table 3, that 0.1 gram. of either the rattlesnake or cobra venom, when given without drugs, produced no symptoms, while the same amount given with opium, salol, and chloroform killed in from four to 30 hours; 0.05 gram., given with drugs, killed in 24 hours; 0.01 gram., with or without drugs, failed to kill, although symptoms were produced in one pig 24 hours after receiving orally 0.01 gram. with drugs.

A series of experiments with the two vegetable poisons, abrin and ricin, with an extract of the poisonous mushroom, *Amanita phalloides*, and with the ptomaines from decayed beef, salmon, fish, and cheese gave negative results. All of these substances appeared to be absorbed, when given, either with or without drugs, to guinea-pigs per stomach, but absorption took place with no degree of uniformity.

The remainder of our work, relative to the absorption of toxins, has to do with the administration of diphtheria toxin to horses. In

all, three horses were treated per mouth with the toxin. The notes on these three cases are as follows:

*Horse 1.*—Brown gelding five years of age, thoroughbred running stock, appetite good, horse normal, except lameness of forelegs. Administered toxin each morning, dose of toxin for each administration being prepared by adding to it 5 c.c. of a saturated solution of salol in chloroform and eight minims of fluid opium. One-quarter grain of atropine sulphate was added and then omitted at times, and we concluded that it was of no apparent benefit. The treatment was given on an empty stomach, water given immediately after treatment, and no food for two or three hours. The toxin used had a strength of 0.5 per L+ dose. The following administrations were made:

August	29, 1906	10 c.c. toxin with drugs
"	30, 1906	25 " " "
"	31, 1906	50 " " "
September	1, 1906	75 " " "
"	2, 1906	100 " " "
"	3, 1906	150 " " "
"	8, 1906	20 " " "

On September 4, 50 c.c. of blood was taken from the animal. A test showed that it contained no antitoxin. On September 14, 17 days after beginning treatment, a sample of blood was drawn from the horse, and on being tested, showed a potency of less than 10 and more than five units per c.c. On September 3 the animal's temperature rose to 105°6 F. and he was listless, ate but little, throat became sore. The temperature remained between 102° and 104° until September 9 when it fell to 100°, rising again on September 11 to 102°. On September 15 the horse died of general paralysis, believed to be due to the diphtheria toxin.

*Horse 2.*—Black gelding, 10 years old, strong, healthy, good appetite; treated with diphtheria toxin No. 08648, prepared as for horse 1; toxin given by special bottle and by stomach tube. Treatment begun on October 1. First dose 4 c.c., increased the dose to 100 c.c. on October 10, and to 225 c.c. on October 19. The animal was bled for sample on October 19 and tests showed that his serum contained nearly 25 units antitoxin per c.c. The animal was not taken off treatment before bleeding for test. On October 29 we began to give the animal a 10 per cent solution of sodium bicarbonate one-half hour before treatment with the toxin. The treatment was continued until November 10, when he received 1,000 c.c. of toxin. The treatment was then discontinued until November 20, when he was bled for test and then received 600 c.c. of toxin.

The test of the serum taken from the horse on November 20 gave a potency of between 50 and 100 units. The amount of toxin administered was increased from 600 c.c. on November 20 until, on Nov. 30, the animal received 1,300 c.c. During the night of November 30 the horse died. Careful post-mortem was made. The liver was very soft, and general pathological conditions, typical of those of horses treated with diphtheria toxins, were found. The temperature curves showed a regularity of response to increased dosage of the toxin as indicated by the temperature.

*Horse 3.*—Brown gelding, seven years old, in good, healthy condition. Began treatment December 12, 1906, with toxin-antitoxin mixture, L+ dose of toxin 0.5 c.c., antitoxin of 300-unit potency; gave drugs in same proportion as in two previous experiments; used fresh serum without preservative; no preservative in toxin.

On December 29, 18 days after beginning treatment, the horse suddenly died from asphyxia. Again we believed that death was produced from diphtheric paralysis. Some blood was collected from the jugular vein of the animal and, on being tested, showed a potency of between 300 and 350 units per c.c. It must be taken into consideration that the blood was taken from this horse and tested while he was receiving daily administrations of toxin, so that some toxin might have been present in the blood.

The above experiments show conclusively that diphtheria toxin, when given orally with drugs, is absorbed in sufficient quantities to produce in the blood of the horse a greater or less amount of antitoxin. The fact that horse 3, which died on the 18th day after treatment was begun, showed a potency of 300 units antitoxin per c.c. in his blood, might possibly indicate that had he been treated more slowly and lived, a high degree of potency would have appeared in his blood.

#### RATE OF ABSORPTION OF TOXIN AND ANTITOXIN FROM THE ALIMENTARY CANAL.

The rate of absorption was studied by the following methods:

1. By flushing out the alimentary canal with a comparatively large dose of sodium sulphate at varying periods of time after administration, per stomach, of diphtheria and tetanus antitoxins to guinea-pigs and diphtheria toxin to rabbits.
2. By the injection of toxin at varying periods of time after the oral administration of the specific antitoxin.
3. By the computation of the amount of unabsorbed antitoxin in the stomach of guinea-pigs at varying periods of time after the antitoxin was placed in the stomach.
4. By the oral administration of antitoxins to children and determination of the antitoxic content of the blood at varying periods of time after treatment.

Tables 4, 5, and 6 give the results of the experiments according to methods 1 and 2, enumerated above.

Eleven guinea-pigs were treated per mouth with 1 grm. dry diphtheria antitoxin, then the alimentary canal was flushed out with sodium sulphate at intervals of time, varying from one to six hours, after the administration. The purpose of this was to remove the unabsorbed antitoxin from the alimentary canal. The pigs were then injected with three fatal doses of toxin. Only two of these pigs died from the toxin, both having been treated with the purgative one hour after

TABLE 4.  
RATE OF ABSORPTION.  
Guinea-pigs, alimentary canal flushed out with sodium sulphate at varying periods of time after administration of antitoxins.

Group	Antitoxin Used Administration per Stomach	No. Fatal Doses of Toxin Injected	No. of Treated Pigs	No. of Con- trol Pigs, All Dead	Periods of Time After Treatment Pigs Were Flushed with Sodium Sulphate	No. of Pigs Saved	Those Pigs Died Which Were Flushed After the Following Periods of Time
1.....	1 grm. dry diph. antitoxin	3	4	1	1, 3, 4, and 6 hours	3	1 hour
2.....	1 grm. dry diph. antitoxin	3	3	1	1½, 2½, and 4 hours	3	0
3.....	1 grm. dry diph. antitoxin	3	2	1	1 and 4½ hours	1	1 hour
4.....	1 grm. dry diph. antitoxin	2	2	1	2 and 3 hours	2	0



receiving the antitoxin. This experiment would indicate that within a little more than one hour a guinea-pig will absorb enough antitoxin from the stomach to protect it against three fatal doses of diphtheria antitoxin.

A glance at Table 5 will show that the same conditions hold true when rabbits are treated per mouth with diphtheria toxin and then flushed out with sodium sulphate one and two hours after the oral treatment.

TABLE 5.  
RATE OF ABSORPTION (*continued*).

Rabbits flushed out with sodium sulphate, per stomach, at varying periods of time after treatment per stomach with large doses of toxin.

No. of Rabbits Treated	No. of Rabbits Flushed Out	Material Used	Periods of Time After Treatment Rabbits Were Flushed Out	Those Rabbits Lived Which Were Flushed After the Following Periods of Time	No. of Rabbits Not Flushed Out All Died
3	2	500 f.d. diph. toxin	1 and 2 hrs.	1 hour	1
3	2	500 " " "	1 and 2 hrs.	1 hour	1*

\* Symptoms two days and recovered, a large, rather old animal.

Eleven pigs, Table 6, received, orally, diphtheria antitoxin and were injected with three fatal doses of the toxin as follows:

One hour before, at the same time, 1, 2, 3, and 24 hours after receiving the antitoxin. Eight of these pigs lived, and three which received the toxin subcutaneously at the same time as the antitoxin orally died from the injection of toxin. This result certainly tends to show that only a short time is necessary for sufficient absorption of antitoxin to take place to protect a guinea-pig against three fatal doses of toxin.

Another series of 11 pigs were treated per stomach with diphtheria antitoxin, then, after periods of time, varying from one to 25 hours, the pigs were killed, stomachs removed, and amount of albumens present determined. One grm. of the serum was given each pig. The amount of albumens present was obtained by precipitation with strong alcohol, filtering, drying the precipitate, and weighing. The weight of the albumens in 1 grm. of the dry serum, prepared as that given to the pigs, was 0.74 grm. We, therefore, used 0.74 grm. albumens as the amount each pig received when computing the results. One of the pigs of this series, No. 4 (110), was killed and stomach contents

TABLE 6.  
RATE OF ABSORPTION.  
Guinea-pigs, injected with toxin at varying periods of time during and after administration of antitoxin.

Group	Antitoxin Administered per Stomach	No. of Fatal Doses of Toxin Injected	No. of Treated Pigs	No. of Control Pigs. All Died	Periods of Time During and After Treatment per Stomach Pigs Were Injected with Toxin	Those Pigs Died Which Were Injected at the Following Times	No. of Pigs Saved
1.....	1 grm. dry diph. serum	3	6	1	1 hour before At same time 1, 2, 3, and 24 hours after	At same time	5
2.....	0.3 grm. dry tetanus antitoxin	2	1	1	3 hours after	0	1
3.....	tetanus antitoxin 40 units	2	4	2	At same time	2	2

washed out as was done in the case of each one of the treated pigs. The albumens from the stomach of this normal pig weighed 0.21 gm. Therefore, assuming that 0.21 gm. was the weight of the albumens from the mucus in the stomach of the treated pigs, we deducted that amount from the total weight of albumens in the computation of the amount of unabsorbed albumens in the stomach of the treated pigs.

These pigs, where not indicated otherwise on the table, were kept without food for 24 hours before treatment and were flushed out with sodium sulphate a few hours before treatment. Each pig, after being anesthetized, was opened through the abdominal wall, the stomach tied off at the pyloric and cardiac ends, and the antitoxin injected into the stomach through a very fine needle. At the time each pig was killed and stomach removed, a few c.c. of blood was collected and tested as to its antitoxic content. In every case the blood serum of these pigs contained some antitoxin and our results obtained by drying and weighing the albumens were confirmed.

Table 7 gives this experiment in detail. From 7.2 to 100 per cent of the antitoxic serum placed in the stomach was absorbed. These results substantiate those obtained by the two former methods. From the stomachs of two pigs there was absorbed, in a period of one hour, in one case 22.9 per cent and in the other 28.3 per cent of the total amount of albumens.

Table 8 gives the antitoxic content of the blood of all the people we have treated with antitoxin per mouth. This, with the period of time after treatment that the blood was collected from the individual, gives a basis for drawing conclusions as to the rate of absorption.

The blood for tests was drawn from these individuals in from 6½ hours to nine days after the oral administration of the antitoxin. A direct comparison cannot be made between results following shorter and longer periods of time for absorption.

Case 17, E. H., 16 years old, absorbed in 6½ hours 0.0269 per cent of the diphtheria antitoxin placed in his stomach, while Case 16, W. K., 16 years old, absorbed in 20 hours only 0.0077 per cent, and Case 11, 15 years old, absorbed 0.025 per cent.

The results point to the conclusion that antitoxin is absorbed in a relatively short time.

TABLE 7.  
RESULTS TABULATED OF PRECIPITATION TESTS, FROM CONTENTS OF STOMACHS OF PIGS TREATED WITH 1 GRM. DRY DIPHTHERIA ANTITOXIN.

No. of Filter	Weight of Filter, Grms.	No. of Guinea-Pigs	Period of Time Prepared Serum Remained in Stomach	Weight of Dried Precipitate and Filter, Grms.	Weight of Dried Precipitate, 0.21 Grm. Deducted	Percentage of Albumens Absorbed	No. of Antitoxic Units Administered	No. of Antitoxic Units Absorbed	Results, Confirmed by Tests of Blood of Animal by Making Guinea-Pig Injections
2.....	3.11	1 (110)	1 hour	3.79	0.57	22.0 per cent	3,000	687	+
3.....	2.75	2 (110)	2 "	3.4	0.30	52.7 "	3,000	1,581	+
4.....	3.07	3 (110)	3 "	3.84	0.50	24.3 "	3,000	720	+
5.....	3.314	5 (110)	20 "	3.52	0	All	3,000	3,000	+
6.....	3.05	6 (110)	25 "	3.21	0	All	3,000	3,000	+
8*	2.82	1 (17)	1 "	3.76	0.53	28.3 per cent	3,000	840	+
9*	2.83	2 (17)	2 "	3.52	0.68	8.1 "	3,000	243	+
10*	3.00	3 (17)	3 "	3.85	0.64	13.5 "	3,000	305	+
11.....	3.22	2 (18)	2 "	4.117	0.687	7.2 "	3,000	216	+

\* Pigs 8, 9, and 10 were not flushed out with sodium sulphate previous to experiment. All others were flushed out before experiment.

TABLE 8.  
DIPHtheria AND Tetanus ANTITOXINS GIVEN PER MOUTH TO MAN.

Case	Age Years	Kind of Antitoxin	No. of Units Antitoxin Administered	No. of Units Absorbed and Con- tained in Total Quantity of Blood of Individual	Time After Administra- tion Blood Was Drawn and Tested	Per cent of Anti- toxin Absorbed, as Shown by Antitoxic Content of Blood
W. P. 1. . . .	7	Diphtheria	6,000	245.0	9 days	0.0408
L. T. C. 2. . .	24	"	13,800	140.0	6 "	0.0101
E. H. 3. . . .	16	"	6,000	189.0	5 "	0.0315
E. S. 4. . . .	7	"	6,000	181.5	82 hours	0.0205
W. E. K. 5. . .	20	"	13,800	283.7	72 "	0.0092
W. K. 6. . . .	15	"	13,800	128.3	72 "	0.0093
H. K. 7. . . .	19	"	4,500	300.0	72 "	0.066
R. S. 8. . . .	7	"	3,000	421.0	24 "	0.14
E. H. H. 9. . .	20	"	3,000	84.0	24 "	0.028
C. V. 10. . . .	17	"	12,000	43.5	22 "	0.0036
E. P. 11. . . .	15	"	3,000	75.2	22 "	0.025
G. L. 12. . . .	15	"	3,000	261.5	22 "	0.087
B. S. 13. . . .	7	"	3,000	562.5	20 "	0.187
A. M. 14. . . .	17	"	3,000	151.5	20 "	0.0505
G. P. 15. . . .	8	"	6,000	750.0	20 "	0.125
W. K. 16. . . .	16	"	12,000	93.2	20 "	0.0077
E. H. 17. . . .	16	"	3,000	80.8	6½ "	0.0269
E. A. 18. . . .	16	Tetanus	1,500	0.0000045	9 days	0.0000003
W. K. 19. . . .	16	"	600	0.000003	72 hours	0.00000005
W. E. K. 20 . .	20	"	1,500	0.0000015	72 "	0.00000001
L. T. C. 21 . .	24	"	900	0.000001	60 "	0.00000001
B. P. 22 . . .	5	"	600	0.000002	48 "	0.00000003
F. G. 23. . . .	7	"	400	0.003418	20 "	0.0000052

#### THE ABSORPTIONS OF DRY ANTITOXINS.

We thought it desirable to see if the antitoxins of diphtheria and tetanus would be absorbed from the stomach equally as well when dry as when liquid. This is of practical importance, should antitoxin ever be administered per mouth because:

1. The dry antitoxin will remain permanent indefinitely.
2. It is very much less expensive to manufacture.
3. It could be kept in stock and in the medicine case and conveniently administered by the physician.
4. The dry antitoxin could be taken with no difficulty or unpleasantness by the patient.

Much of the work in the experiments already described has been done with the dried serums. Table 9 gives the results of the administration of dry antitoxin to guinea-pigs, per mouth. In Table 9 it is shown that 46 out of 53 pigs, treated with dry diphtheria antitoxin, were saved from death after the injection of three fatal doses of toxin. Thirteen control pigs all died in less than four days. Likewise, out of 18 pigs treated per mouth with tetanus antitoxin, only one was lost after the series were injected with 2 and 2.5 fatal doses of tetanus



toxin. We can safely state that the dry antitoxin is absorbed equally as well as the liquid serum.

TABLE 9.  
DRY DIPHTHERIA ANTITOXIN, PER STOMACH, GUINEA-PIGS.

Group	Amount in Grms. Dry Serum Given	No. of Fatal Doses of Toxin In- jected	No. of Pigs in Group	No. of Con- trol Pigs in Group All Died	No. of Treated Pigs in Group	No. Treated Pigs Saved	No. Treated Pigs Lost
3.....	0.5	3	7	2	5	5	0
4.....	I	3	3	I	2	2	0
6.....	I	3	6	I	5	4	I*
7.....	I	3	4	I	3	3	0
9.....	I	3	9	I	8	7	I*
10.....	I	3	8	I	7	6	I*
11.....	0.1 and 0.3	3	3	I	2	2	0
12.....	0.05 to 0.2	3	6	I	5	4	I
13.....	0.01 to 0.05	3	6	I	5	4	I
14.....	0.03 to 0.05	3	4	I	3	2	I
15.....	0.03 to 0.5	3	10	2	8	7	I
						46	7

DRY ANTITETANIC SERUM, PER STOMACH, GUINEA PIGS.

2.....	I	2	4	I	3	3	0
3.....	0.2 to 0.3	2	4	I	3	3	0
4.....	I	2	5	I	4	4	0
7.....	0.1 to 0.3	2.5	4	I	3	2	I
8.....	0.1 to 0.5	2.5	6	I	5	5	0
			23	5	18	17	I

\* These pigs were used in testing rate absorption and were flushed out one hour after giving the antitoxin per stomach.

#### ORAL ADMINISTRATION OF ANTITOXINS IN MAN.

Since the publication of our last report experiments have been continued with diphtheria and tetanus antitoxins, relative to their administration per mouth and absorption in children. In that paper we reported the results of the oral administration of the antitoxin to a series of 14 persons, whose ages were from five years to 29 years. From 3,000 to 13,800 units of diphtheria and 600 to 1,500 units of tetanus antitoxins were given to these individuals per mouth. Nine of these 14 persons received, orally, the diphtheria antitoxin, and guinea-pig tests demonstrated the presence of antitoxin in the blood in amounts which averaged 0.05 unit antitoxin per c.c. of the total blood in the body, while the five who were given the tetanus antitoxin averaged 0.0000024 unit per c.c. of total blood. Owing to the insufficient quantities of blood to carry on complete tests, the maximum amount of antitoxin was not demonstrated except in three of the cases. Therefore the above averages do not represent the maxi-

imum amount of antitoxin that was absorbed after the oral administrations.

Five individuals, who received subcutaneous injections of diphtheria antitoxin in quantities of from 320 to 1,040 units, showed, by guinea-pig tests, an average of 0.19 unit per c.c. of body blood. It was impossible, however, to make a direct comparison of the two methods because the maximum amount of antitoxin absorbed after the oral administration was not determined.

In the present work we have endeavored to reach definite conclusions, by making a direct comparison of the two methods. The following series of experiments were conducted in the same manner as described in our former report:

The individual under treatment was bled from the median cephalic vein before treatment with the antitoxin. The serum was collected from this blood and, together with a given number of fatal doses of toxin, was injected subcutaneously into guinea-pigs. Each individual under treatment then was either treated per mouth or was injected subcutaneously with a given amount of antitoxin and, after a few hours, the blood was again collected as before and tested as to its protective influence against the toxin in question, by injecting guinea pigs with the blood serum and toxin. A comparison of these results with those from injections of the human blood serum, before the individual was treated per mouth or subcutaneously, indicated whether the antitoxin which had been administered to the individual had resisted digestion and had been absorbed in sufficient quantities to cause the protective antitoxin to be present in the blood of the individual. The maximum amount of absorbed antitoxin, as indicated by the antitoxic content of the blood, was determined in each case. Tables 10 and 11 give in detail the results of these experiments.

In Tables 10 and 11, in the second column, under "guinea-pig injections" etc., the + and - signs indicate the range within which the toxic dose for a 250 gm. pig was determined. As, for example, Case 1 (E. H.), Table 10, the amount of diphtheria toxin necessary to kill a 250 gm. pig which was injected with 0.2 c.c. of the blood serum from E. H. after treatment with antitoxin per mouth lies between 12 and 15 fatal doses. In determining the amount of antitoxin absorbed, however, the number of fatal doses which was known to be neutralized by the given amount of human blood serum was used as a basis of computation in finding the number of units of antitoxin per c.c. in the blood of the individual. Thus, in the above illustration, Case 1 (E. H.), 12 times the fatal dose of toxin was taken as the maximum amount of toxin a 250 gm. pig would withstand when protected by 0.2 c.c. of the serum of E. H.

In Table 11 the average absorption per c.c. of body blood should be taken from Cases 1 to 8 in order to make a direct comparison with the average absorption of the eight cases, treated orally, given in Table 10. The reason for this is that no check tests were made for the antitoxic content of the serum of Cases 9 to 13 (Table 11) before injection with the antitoxin. Therefore, the antitoxic content of the blood of Cases 9 to 13, as indicated on the table, are probably, in reality, considerably less. A glance at Cases 1 to 8, in both Tables 10 and 11, will show that normal human serum, before the administration of diphtheria antitoxin, gives some protection against the toxin. The degree of this protection varies within comparatively wide limits.

The eight individuals who received the serum per mouth (Table 10) were treated as follows:

One-half hour before taking the serum, the individual drank a half-glass full of a 1 per cent solution of sodium bicarbonate. The dry antitoxin was dissolved in a sufficient amount of water and to it was added one minim of fluid opium (containing 22 grains crystallized morphine in each fluid ounce, and four times stronger than Tincture opium U. S. P.), and 4 to 10 minims of a saturated solution of salol in chloroform.

Assuming that the oral method of administration is of advantage as a prophylactic in children, we are confronted with the question, What is the most convenient and practical form in which to administer the serum and drugs? We are convinced that antitoxin may be used in the dry form. How the necessary drugs should be combined with the serum and whether the preparation should be given to the patient in the dry form inclosed in a capsule or made into a tablet, or whether it should be dissolved in water, are questions for the clinician.

A careful comparison of the two methods of administration and results of the actual trial, as shown in Tables 10 and 11, demonstrates that the oral treatment with antitoxin is effective. Our experiments warrant us in concluding that, in the case of children, the oral administration is followed by rapid absorption, almost as complete absorption as that following the hypodermic injection. Moreover, we do not hesitate in stating that the oral method is safer than the hypodermic method of administration. We have not observed any untoward effects in a single one of the 24 cases that we have treated orally.

A study of Table 12 shows the number of antitoxic units that should theoretically have appeared in the blood and the number of

TABLE 10.  
DIPHTHERIA ANTITOXINS, PER MOUTH, MAN.

CASE	SEX	AGE	ANTITOXIN I gm., Diphtheria	No. UNITS ANTITOXIN ADMINIS- TERED	GUINEA-PIG INJECTIONS BEFORE ADMINISTRATION OF ANTITOXIN TO FIND HOW MUCH PROTECTION, IF ANY, EXISTED IN THE HUMAN SERUM. ALL PIGS DIED		GUINEA-PIG INJECTIONS AFTER ADMINISTRATION OF ANTITOXIN SHOWING MAXIMUM PROTECTION IN BLOOD OF INDIVIDUAL. ALL PIGS LIVED		RESULTS		
					No. c.c. of Human Serum	No. Fatal Doses Toxin	No. c.c. of Human Serum	No. Fatal Doses Toxin	Period of Time After Adminis- tration Test Was Made for Anti- toxin Content of Blood	No. Units Antitoxin in 1 c.c. of Blood	No. Antitoxin Units Absorbed and Contained in Total Quantity of Blood
E. H. 1.....	Male	15		3,000	0.5	10	0.5	{ -15 + 12	6½ hours	0.04	74.6
G. T. 2.....	"	15	"	3,000	0.5	18	0.5	{ -35 + 30	22 "	0.24	261.5
E. P. 3.....	"	15	"	3,000	0.5	3	0.5	{ -10 + 5	22 "	0.04	75.2
C. V. 4.....	"	17	"	3,000	1	3	0.5	{ -5 + 4	22 "	0.02	43.5
W. K. 5.....	"	16	"	3,000	0.5	3	0.5	{ -6 + 5	20 "	0.04	93.2
G. P. 6.....	"	8	"	3,000	0.5	10	0.5	{ -35 + 30	20 "	0.4	750
B. S. 7.....	"	7	"	6,000	0.5	12	0.2	{ -25 + 18	20 "	0.3	569.5
A. M. 8.....	"	17	"	3,000	0.5	3	0.5	{ -6 + 5	20 "	0.02	151.5
Average										0.1375	251.5

TABLE II.  
DIPHTHERIA ANTITOXIN, SUBCUTANEOUS INJECTION, MAN.

CASE	SEX	AGE	WEIGHT GRAMS.	ANTI- TOXIN	No. UNITS ANTITOXIN INJECTED	GUINEA-PIG INJECTIONS BEFORE INJECTION OF ANTITOXIN TO FIND HOW MUCH PROTECTION, IF ANY, EXISTED IN THE HUMAN SERUM. ALL PIGS DIED.		GUINEA-PIG INJECTIONS AFTER INJECTION OF ANTITOXIN SHOWING MAXIMUM PROTECTION IN BLOOD OF TREATED INDIVIDUALS. ALL PIGS LIVED		PERIOD OF TIME AFTER INJECTION TEST WAS MADE FOR ANTITOXIC CONTENT OF BLOOD	No. OF ANTITOXIC UNITS IN 1 C. C. OF BLOOD	No. OF ANTITOXIC UNITS AB- SORBED AND CONTAINED IN TOTAL QUANTITY OF BLOOD
						No. c.c. Human Serum Injected	No. Fatal Doses of Toxin	No. c.c. of Human Serum	No. Fatal Doses of Toxin			
E. K. 1	Male	7	20.515	1 gram	3,000	0.2	15	0.2	{ -35 +30 }	24 hours	0.75	1280.7
L. 2	"	6	14.547	1 "	3,000	0.2	20	0.2	{ -35 +30 }	24 "	0.5	606.1
E. 3	"	8	21.634	$\frac{1}{2}$ "	1,500	0.2	10	0.2	{ -18 +10 }	72 "	0.3	540.8
H. 4	"	8	16.030	$\frac{1}{2}$ "	1,500	0.2	6	0.2	{ -7 +6 }	6 "	0.05	66.7
N. 5	"	9	26.110	1 "	3,000	0.2	18	0.2	{ -25 +20 }	20 "	0.1	217.5
W. 6	"	7	17.004	1 "	3,000	0.2	7	0.2	{ -10 +7 }	20 "	0.05	74.6
B. 7	"	8	20.515	1 "	3,000	0.2	7	0.2	{ -20 +15 }	20 "	0.4	683.8
H. A. 8	"	10	22.500	2 "	6,000	0.2	8	0.2	{ -10 +14 }	20 "	0.3	562.5
Five cases, the antitoxic properties of blood before injection not determined												
F. 9	Male	7	27.272	Liquid	1,040	Not determined		1	{ -20 +12 }	Average	0.366	
B. 10	"	7	24.500	"	1,040	"	"	1	{ -12 +7 }	24 hours	-0.2	454.7
H. 11	"	7 $\frac{1}{2}$	27.000	"	480	"	"	1	{ -12 +7 }	15 days	-0.12	244.9
E. 12	"	7	23.181	"	320	"	"	1	{ -15 +14 }	24 hours	-0.12	270
B. Y. 13	"	7	23.501	"	1,040	"	"	1	{ -38 +30 }	24 "	-0.15	280.7
										24 "	-0.38	747.7
										Average	0.104	

units that actually was found in the case of those individuals who received subcutaneous injections of the antitoxin. The average number of units absorbed and present in the blood of the eight individuals treated per mouth (Table 10) was 0.1375 unit per c.c. The average number of units per c.c. of blood of those individuals who received the subcutaneous injection of the antitoxin was 0.306. The ages of those who received the oral administrations varied from seven to 17 years, there being only two cases who were under 15 years of age. On the other hand, those who received the hypodermic treatment were all under 10 years of age. If all of the eight cases treated per mouth had been children under 10 years of age, we would expect to find a larger average amount of antitoxin absorbed per c.c. of the body blood. This fact gives the advantage, in making this direct comparison, to the subcutaneous method. Unfortunately, at the time we made the test of the oral method, as indicated in Table 10, we were unable to secure many children under 10 years. As the results of this comparison stand, however, we feel sure that, under proper conditions, enough antitoxin is absorbed from the stomach of a child to protect the child against subsequent infection.

TABLE 12.  
SUBCUTANEOUS INJECTION, DIPHTHERIA ANTITOXIN, MAN.

Relation of amounts actually absorbed, as shown by antitoxic content of the blood, to amounts which, theoretically, should have been absorbed.

Case	Weight Grms.	No. of Units Antitoxin In- jected Subcuta- neously	No. of Units That Should Theoretically Have Been Ab- sorbed per c.c. in Body Blood	No. of Units per c.c. Actu- ally Absorbed and Present in Body Blood	No. of Units per c.c. not Absorbed	Percent of Antitoxin Ab- sorbed per c.c. Body Blood
E. K. 1	20.515	3,000	1.75	0.75	1	42
L. 2	14.547	3,000	2.47	0.5	1.97	20
E. 3	21.034	1,500	0.82	0.3	0.52	36
H. 4	16.080	1,500	1.12	0.05	1.07	4
N. 5	26.110	3,000	1.37	0.1	1.27	72
W. 6	17.004	3,000	2.01	0.05	1.96	2
B. 7	20.515	3,000	1.75	0.4	1.35	22
H. A. 8	22.500	6,000	3.17	0.3	2.87	9
F. 9	27.272	1,040	0.46	0.2	0.26	43
B. 10	24.500	1,040	0.50	0.12	0.38	24
H. 11	27.000	480	0.21	0.12	0.00	57
E. 12	23.181	320	0.16	0.15	0.015	90
B. Y. 13	23.591	1,040	0.52	0.38	0.14	73
Average	21.054	2,147	1.25	0.26	0.99	38

#### CONCLUSIONS.

1. Toxins and antitoxins when given by mouth are usually rendered inert by the digestive processes. Their therapeutic or immunizing value is uncertain and not to be relied upon.



2. If digestion is inhibited, which may be readily accomplished by the use of appropriate drugs, toxins and antitoxins are absorbed unchanged and apparently in sufficient quantity and with such uniformity as to warrant the use of this method for therapeutic and immunizing purposes.

3. In treating children with antitoxin per mouth, the following method has given uniform and satisfactory results. One-half hour before administering the serum the child is given one glass of 1 per cent sodium bicarbonate solution. When the antitoxin is given there is added to it one minim of Fl. Ext. Opii and from four to 10 minims of saturated solution of salol in chloroform. When possible no food should be given for at least four hours before administering the serum.

4. In the 19 children and the hundreds of animals used in these experiments, there was no evidence of any "serum sickness" or anaphylaxis.

5. In our opinion the oral method of administering antitoxins of tetanus and diphtheria is the preferable one for prophylaxis.

*a.* On account of the absence of danger and the ease of administration.

*b.* Because the cost may be very materially lessened.

6. The hypodermic method of administering sera for curative purposes is the only one to be recommended unless extensive clinical experience should show that the oral method is equally efficacious.

7. A relatively high degree of immunity may be produced in animals by the oral administration of toxins if the absorption of the same is promoted by such means as we have suggested.

## ON THE MECHANISM OF OPSONIC ACTION.\*

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### ACTIVATION OF HEATED OPSONIC SERUM.

NUMEROUS investigators have found that the total opsonic action of fresh serum is the result of the combined action of two distinct bodies, one thermostable and the other thermolabile. In other words, opsonins have the same general constitution as lysins. There is also practically general agreement to the effect that the thermostable body, i.e., the body which persists after serum has been heated to 55°C. or so for about 30 minutes and is absorbable at 0°C., by itself is capable of opsonic action. Heated normal serum, however, contains comparatively little of thermostable opsonic substance, whereas immune serum may be very rich in this body. Driven by the conception that opsonins are different from the tropins (as the substances that promote phagocytosis in immune serum have been called) Neufeld<sup>1</sup> would deny that heated normal serum has any power to promote phagocytosis except in special instances. This is not in accord with the observations with which I am familiar as mixtures containing heated normal serum (58–60°C. for 30 min.) have always given greater phagocytosis than mixtures containing salt solution only. It is true that accurate numerical methods sometimes may be necessary to detect the difference, but the difference is there and unmistakable. It should be stated that according to Hamilton's<sup>2</sup> investigations the opsonins for certain pseudodiphtheria bacilli in the serum of rabbits and goats, normal as well as immune, are simple thermostable substances, those in immune serum consequently corresponding closely to Neufeld's bacteriotropins. In Eggers's<sup>3</sup> hands the serum from some persons proved to contain inactivable thermostable opsonic elements at least with respect to the pneumococcus and streptococcus. This observation is of interest as it may explain why the earlier

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<sup>1</sup> Kolle and Wassermann's *Handbuch*, 1908, Ergänzungsband 2, p. 313.

<sup>2</sup> *Jour. Infect. Dis.*, 1908, 5, p. 570.

<sup>3</sup> *Ibid.*, 1908, 5, p. 263.

attempts by Hektoen and Ruediger<sup>1</sup> and Bullock and Atkin<sup>2</sup> at re-activation proved failures.

Most of the experiments the results of which have led to the conclusion that two substances co-operate in producing the opsonic effect of many sera have been made with bacteria. Neufeld and Bickel<sup>3</sup> have demonstrated well the interaction of thermostable and thermolabile substances in the production of phagocytosis of red corpuscles, but they interpret this opsonic effect as caused by the lytic amboceptor and complement. Consequently further results obtained with red corpuscles as the object of opsonification may be of interest. In my experiments the mixtures contained equal quantities of 5 per cent suspension of carefully washed red corpuscles, of suspension of carefully washed leucocytes obtained from pleural exudate in a dog, produced by aleuronat, and of serum + salt solution, the total quantity being always the same. After one hour at 37° C., during which the tubes containing the mixtures were gently agitated by means of a shaking machine, smears were made and stained and the percentage of phagocytic leucocytes determined by counting a large number of leucocytes (100-400). Usually a larger percentage of mononuclears are phagocytic than of the polymorphonuclears, but unless otherwise stated the figures represent the gross percentage of phagocytic cells among all forms of leucocytes. The method, while by no means perfect, readily detects accurately enough material differences in the amount of phagocytosis in comparable mixtures. A brief statement of the result shown in Table 1 may be desirable: In doses of 0.001 c.c. the heated serum of a rabbit immunized to goat blood corpuscles proved only feebly opsonic for washed goat corpuscles, using dog leucocytes as phagocytes, but the addition of 0.01 c.c. of normal guinea-pig serum, of itself without demonstrable opsonic action, materially increased the amount of phagocytosis.

TABLE 1.  
PHAGOCYTOSIS OF GOAT CORPUSCLES UNDER THE INFLUENCE OF IMMUNE RABBIT SERUM AND NORMAL GUINEA-PIG SERUM.

Immune Serum	Normal Guinea-Pig Serum	Phagocytosis
0.001		4
0.001	0.01	20
	0.01	0

<sup>1</sup> *Ibid.*, 1905, 2, p. 120.

<sup>3</sup> *Arch. kais. Gesundh.*, 1907, 27, p. 310.

<sup>2</sup> *Proc. Roy. Soc.*, 1905, 74, p. 380.

Other illustrations of the same phenomenon are given in Tables 2, 3, 4, and 5. The tables show that the activating or stimulating element may be contained in the serum of some other animal than that furnishing the thermostable body. Table 5 gives an instance of this phenomenon on part of a normal serum (guinea-pig). Elsewhere<sup>1</sup> are given similar results obtained with heated and fresh dog serum and rat corpuscles.

TABLE 2.

PHAGOCYTOSIS OF HUMAN CORPUSCLES UNDER THE INFLUENCE OF IMMUNE (ANTHUMAN) RABBIT SERUM AND NORMAL DOG SERUM.

Immune Serum	Normal Dog Serum	Phagocytosis
0.0013	.....	16
0.0004	.....	7
0.0013	0.02	50
0.0004	0.02	25
.....	0.02	4

TABLE 3.

PHAGOCYTOSIS OF HUMAN CORPUSCLES UNDER THE INFLUENCE OF IMMUNE (ANTHUMAN) RABBIT SERUM AND NORMAL HUMAN SERUM.

Immune Serum	Normal Human Serum	Phagocytosis
0.05	.....	2
0.05	0.012	9
0.05	0.006	10
.....	0.012	1
.....	0.006	0

TABLE 4.

PHAGOCYTOSIS OF RABBIT CORPUSCLES UNDER THE INFLUENCE OF IMMUNE AND NORMAL DOG SERUM.

Immune Serum	Normal Serum	Phagocytosis
0.012	.....	15
0.004	.....	8
0.012	0.02	35
0.004	0.02	10
.....	0.02	3

TABLE 5.

PHAGOCYTOSIS OF GOAT CORPUSCLES UNDER THE INFLUENCE OF HEATED AND UNHEATED GUINEA-PIG SERUM.

Heated Serum (60° 30 min.)	Fresh Serum	Phagocytosis
0.05	.....	0
0.025	.....	0
0.05	0.012	17
0.025	0.012	8
.....	0.012	0

<sup>1</sup> Hektoen, *Jour. Infect. Dis.*, 1908, 5, p. 249.

The results of these experiments indicate that many hemopsonic sera, normal as well as immune, owe their full action to a thermostable opsonic substance and a thermolabile complement-like body which greatly promotes the action of the first substance. The results also suggest the query, why, if the opsonic action of normal serum really is produced by lytic amboceptor and complement as claimed by Neufeld, should we not consider the opsonic action of immune serum as owing to the lytic amboceptor and complement? So far as results of activation experiments go, normal and immune sera act alike, and the logical conclusion would seem to be that in both cases the opsonic action is the effect of the same bodies, be they identical or not with the lytic amboceptor and complement, and not of dissimilar bodies, as Neufeld would have it when he urges that the hemotropic power of normal serum be ascribed to the lytic amboceptor and complement and that of immune serum at least to a very large extent to a different kind of substance without any normal homologue, namely hemotropin. Neufeld assumes that the so-called cytotropins cannot be activated, and when he finds that small quantities of heated immune serum in itself without opsonic effect is activated by normal serum, he seems to make the further assumption that in this case too it is the lytic amboceptor which is activated. The theory meets difficulty too when we attempt to apply it to opsonic substances for bacteria like pneumococci and streptococci that are not subject to lysis by serum but readily opsonified. Neither does Neufeld's contention harmonize well with the fact that on the entrance of antigen into the body there occurs, as I have shown, a depression of the opsonic index which is specific for the antigen in question.<sup>1</sup> At present it seems to me the other view, namely that the specific opsonic substances in normal and immune serum are identical, differing only in quantity, harmonizes better with the established facts in regard to these substances as well as in regard to antibodies in general. Hamilton's simple thermostable opsonic substances correspond well to Neufeld's tropins but they occur in normal as well as immune serum.<sup>2</sup>

<sup>1</sup> *Ibid.*

<sup>2</sup> Wright and Reid (Wright and Reid, *Proc. Roy. Soc.*, 1906, 77, p. 211) make objection to the term bacteriotropin as used by Neufeld, in the first place because better applicable to the whole class of substances which combine with bacteria and secondly because used by Wright in another sense from Neufeld's as early as 1899.

## MECHANISM OF OPSONIC ACTIVATION.

The question arises, how does activation of heated opsonic serum take place? Is it possible to show by appropriate absorption experiments whether or not the thermolabile body is anchored to the corpuscle (or, in case of bacteriopsonein, to the bacterium) by means of the thermostable body, or perhaps in some other way? To this end the following experiments were devised. The consideration upon which they are based is this—that if the opsonic complement is fixed firmly to the corpuscle or bacterium its activating influence should persist after the corpuscle or bacterium treated with the sera in question is washed in salt solution. The first two experiments constitute an analysis in this way of the action of small quantities of normal serum;

TABLE 6.

ANALYSIS OF ACTION OF FRESH DOG SERUM IN PROMOTING PHAGOCYTOSIS OF RAT CORPUSCLES UNDER THE INFLUENCE OF HEATED DOG SERUM.

	Corpuscles (5% susp.)	Heated Dog Serum (50° 30 min.)	Fresh Serum (same as the heated)	NaCl Sol.
Tube 1.....	0.5 c.c.	0.1 c.c.	....	0.1 c.c.
Tube 2.....	0.5	0.1	0.03	0.07
Tube 3.....	0.5	1.0	....	0.1
Tube 4.....	0.5	...	....	0.2

After inoculation at 37° C. for 60 min. the corpuscles were washed twice in large quantities of NaCl solution and in each case there were added suspension of washed leucocytes 0.5 c.c. and NaCl sol. 0.1 c.c. **except that Tube 3 received fresh normal serum 0.03 in NaCl sol. 0.07 in place of NaCl sol. only.** After incubation under gentle shaking for 60 min. smears were made and the percentage of mononuclears determined with the following result:

Tube 1.....	1
Tube 2.....	1
Tube 3.....	50
Tube 4.....	0

TABLE 7.

ANALYSIS OF THE ACTION OF NORMAL GOAT SERUM IN PROMOTING PHAGOCYTOSIS OF SHEEP CORPUSCLES UNDER THE INFLUENCE OF IMMUNE GOAT SERUM.

	Corpuscles (5% Susp.)	Immune Serum (60° 30 min.)	Fresh Serum	NaCl Sol.
Tube 1.....	0.2 c.c.	0.0025 c.c.	....	} In all sufficient to make the total quantity 0.4 c.c.
" 2.....	"	"	0.01 c.c.	
" 3.....	"	"	0.005	
" 4.....	"	"	....	
" 5.....	"	.....	....	
" 6.....	"	.....	....	
" 7.....	"	.....	....	



After incubation at 37°C. for 60 min. the corpuscles were washed carefully, the supernatant fluid removed wholly, and the experiment completed by adding to each tube as follows, and incubating for one hour:

Tube 1.	NaCl sol.	0.1	+	suspension of dog leucocytes	0.2	.....	24*
" 2.	"	0.1	+	"	"	0.2	..... 22
" 3.	"	0.1	+	"	"	0.2	..... 20
" 4.	Nor. ser.	0.01		in NaCl sol.	0.1	+	susp. dog. leuc. 0.2 ..... 50
" 5.	"	0.005	"	"	0.1	+	" " " 0.2 ..... 44
" 6.	"	0.01	"	"	0.1	+	" " " 0.2 ..... 0
" 7.	"	0.005	"	"	0.1	+	" " " 0.2 ..... 0

\* The figures give the percentage of mononuclears engaged in phagocytosis.

TABLE 8.

ANALYSIS OF ACTION OF NORMAL HUMAN SERUM IN PROMOTION OF PHAGOCYTOSIS OF COLON BACILLI UNDER INFLUENCE OF HEATED IMMUNE HUMAN SERUM.

	Bacillary Susp.	Immune Serum	Normal Serum	NaCl Sol.
Tube 1.....	0.3 c.c.	0.1 c.c.	.....	} In all sufficient to make the total quantity 0.5 c.c.
" 2.....	0.3	0.1	0.02 c.c.	
" 3.....	0.3	0.1	.....	
" 4.....	0.3	0.1	0.02	

After incubation for 45 minutes at 37° the bacteria were removed by long centrifugation, washed, resuspended in 0.3 NaCl solution, and in each case there were added suspension of leucocytes 0.1 cc. and NaCl 0.1 c.c. **except that Tube 3 received normal serum 0.02 in NaCl 0.08 in place of NaCl solution only.** After incubation for 30 minutes smears were made and the average number of bacilli taken up in each case determined in the usual way and with the following result:

Mixture 1.....	4.
Mixture 2.....	3.8
<b>Mixture 3.....</b>	<b>11.</b>
Mixture 4.....	0.0

the third experiment, by Dr. D. J. Davis, gives the result of a similar analysis of the promotion by normal human serum of the opsonic effect of immune human serum on a colon bacillus.

The results indicate clearly that opsonic complement is not fixed firmly to the bacterium or corpuscle. The thermostable opsonic element, however, is fixed more firmly as activation is obtainable after washing. Hence the removal of opsonin that Sellards<sup>1</sup> and Centanni<sup>2</sup> obtained by washing treated bacteria may have been more apparent than real, the loss in the amount of phagocytosis being accountable by the removal of the activating substance which appears to remain quite free in the fluid of the phagocytic mixtures. If that be the case this fluid should retain activating properties after the corpuscles or

<sup>1</sup> *Jour. Infect Dis.*, 1908, 5, p. 308.

<sup>2</sup> *Ztschr. f. physiol. Chemie*, 1908, 55, p. 140.

bacteria treated with opsonic serum are removed. Such is the case at least so far as mixtures of dog serum with rat corpuscles are concerned. Indeed, in mixtures of dog serum and corpuscles, dog serum and leucocytes, as well as of serum, corpuscles, and leucocytes, incubated at 37° for 45-60 min., the supernatant fluid upon proper dilution is strongly activating, sometimes fully equivalent in its activating power to that of corresponding amounts of fresh unused serum.

TABLE 9.  
THE ACTIVATING POWER OF THE FLUID IN INCUBATED MIXTURES OF SERUM, CORPUSCLES, AND LEUCOCYTES

Mixtures.		Percentage of Phagocytic Mononuclears
Each contained 0.2 c.c. 5% susp. rat corp., 0.02 c.c. susp. dog leucocytes, sera as shown below; the total quantity always made 0.6 c.c. by NaCl solution		
Heated Serum 0.05	.....	14
Heated Serum 0.05 + Fluid 1	} Amount of serum in each fluid = 0.005 c.c.	45
Heated Serum 0.05 + Fluid 2		50
Heated Serum 0.05 + Fluid 3		49
Heated Serum 0.05 + Normal Serum 0.005 c.c.		52
Fluid 1	} Amount of serum in each fluid = 0.005 c.c.	0
Fluid 2		0
Fluid 3		0
Normal Serum 0.005 c.c.		0

NOTE.—Fluid 1 is requisite amount of fluid in a mixture of 0.2 c.c. 5 per cent susp. of corpuscles + normal serum 0.05 c.c. + NaCl sol. 0.15 c.c., incubated at 37° C. for 1 hr. Fluid 2 is requisite amount of fluid in a mixture treated in the same way but with susp. dog. leuc. in place of corpuscles. Fluid 3 is requisite amount of fluid in a mixture of 0.1 c.c. 10 per cent susp. of dog corpuscles + leucocytic susp. 0.1 c.c. + normal serum 0.05 + NaCl sol. 0.15, incubated at 37° C. for 1 hr.

On heating the supernatant fluid the activating property is lost.

In this particular case then there is reason to conclude that the complementing or activating element is not fixed either by the corpuscle, by the phagocytes, or by the phagocytes when engaged in taking up the corpuscles. Further experiments designed to reveal something further of the mechanism of activation have not given decisive results and no indication of any direct stimulating effect upon the phagocytes to the extent that it will survive washing in salt solution has been obtained.

#### THE EFFECT OF HEAT UPON MIXTURES OF BACTERIA AND OPSONIC SERUM.

If the activating element remains quite free in the fluid of the phagocytic mixture, one would expect that heating the mixture to 60° C. for 15-30 min. before adding the leucocytes will destroy this element so that the amount of phagocytosis obtained on adding

leucocytes after heating practically would be the same as that obtained on using heated serum from the start.

In their original experiments on the opsonic action of serum, Wright and Douglas<sup>1</sup> found that if the mixture of staphylococci and serum was heated immediately after it was made only a minimal phagocytosis would result. But if the mixture was first digested for 15 minutes at 37° C. and then heated for 15 min. or longer at 60° C. pronounced phagocytosis would result. Bullock and Atkin,<sup>2</sup> Sellards,<sup>3</sup> and others have obtained confirmatory results, the phagocytosis on heating after digestion at 37° C. being in some cases at least somewhat less than in the unheated control mixture. Bullock and Atkin found that after the opsonin in normal serum had united with staphylococci, the mixture of serum and cocci could be heated to 60° C. for several hours without abolition of the opsonic effect. Using serum in dilution of 1:10 Sellards, however, noted a marked reduction in phagocytosis when the mixture of serum and staphylococci was heated after digestion, though not quite so marked as when heated before the digestion. All these experiments were made with human serum and staphylococci. In some of them, notably Bullock and Atkins, very dense suspensions of staphylococci were used. The possibility also suggests itself that the serum in some trials may have contained newly formed thermostable opsonic elements as the product of unrecognized staphylococcus infection. For these and other reasons further observations are desirable and the results of some new experiments are given in Table 10.

TABLE 10.

THE EFFECTS ON THE OPSONIC ACTION OF HEATING MIXTURES OF SERUM AND BACTERIA.

TREATMENT OF SERUM + BACTERIA BEFORE ADDING LEUCOCYTES	PHAGOCYTOSIS			
	Staphylo- coccus	Strepto- coccus	Pneumo- coccus	Anthrax Bacillus
At 58° for 30 min., then at 37° 30 min. ....	4.	0.2	1.2	35
At 37° for 30 min., then at 58° 30 min. ....	6.3	0.5	1.8	37
At 37° for 60 min. ....	12.2	5.	4.	75

NOTE.—The figures represent the number (average) of bacteria per leucocyte in mixtures of equal parts of human serum, bacterial suspension, and leucocytes, except those under Anthrax Bacillus, which represent percentage of phagocytic polynuclears in mixtures containing dog serum and dog leucocytes.

<sup>1</sup> *Proc. Roy. Soc.*, 1903, 72, p. 357.<sup>3</sup> *Jour. Infect. Dis.*, 1908, 5, p. 308.<sup>2</sup> *Ibid.*, 1905, 74, p. 380.

When mixed with dog serum rat corpuscles commonly undergo more or less lysis if placed at 58–60° C. for 15–60 min. Consequently experiments like those just given were made at low temperatures. At 45° no difference could be made out between the more highly heated mixtures and that left at 37°; at 54°, however, the following results were obtained:

1. Serum + corpuscles at 54° 30 min. and then at 37° 30 min.....	22
2. Serum + corpuscles at 37° 30 min. and then at 54° 30 min.....	25
3. Serum + corpuscles at 37° 60 min.....	65

In all these trials the phagocytosis is greatly diminished in the heated mixtures. While the diminution is not quite as marked in the mixtures heated after incubation as in those heated before, yet the results harmonize well with the theory that the activating element remains free in the phagocytic mixture. If this explanation of the facts noted proves to be correct it will establish a fundamental distinction between the mechanism of opsonification and of lysis, because in the latter process it is held that the complement is bound by the amboceptor. Meyer<sup>1</sup> also suggests the possibility of this distinction.

#### THE FIXATION OF THE THERMOSTABLE OPSONIC ELEMENT BY THE OBJECT OF ITS ACTION.

Because of its bearing upon the opsonic theory itself of the question of fixation of opsonin by the object of phagocytosis I have made a number of additional experiments especially with rat corpuscles and dog serum.

In a phagocytic mixture containing 0.2 c.c. of a 5 per cent suspension of carefully washed rat corpuscles, 0.05 c.c. fresh normal dog serum + 0.15 c.c. NaCl solution, and 0.2 c.c. of suspension of dog leucocytes (washed), usually about 40–50 per cent of the mononuclear leucocytes are found to be engaged in phagocytosis after an incubation of 45–60 min. Merely pouring off the supernatant fluid after incubating the corpuscles with serum + salt solution before adding the leucocytes reduces the amount of phagocytic mononuclear cells to 10 or 12 per cent but this reduction is not always materially increased even if the corpuscles after treatment with serum are washed five to eight times or more in many hundred times their volume of salt solution.

<sup>1</sup> *Berl. klin. Wchnschr.*, 1908, 45, p. 951.

With dog serum heated to 60° C. for 30 min. the initial phagocytosis before washing is about 10-15 per cent. On the addition of 0.005 c.c. of fresh dog serum to corpuscles treated with heated serum at the same time as leucocytes are added the usual amount of phagocytosis given by unheated serum (0.05 c.c.) is often obtained no matter whether the treated corpuscles are washed or not before adding the serum. Approximately the same result is obtained on adding 0.005 c.c. of normal serum to corpuscles treated with normal serum and then washed, indicating that in either case the corpuscles became sensitized to about the same degree by taking up the heat-resistant opsonic element. Sometimes only little phagocytosis is obtained with corpuscles treated with serum and then washed several times in salt solution, but even in such cases the addition of 0.005 c.c. normal serum has given pronounced activation as shown in the results of the experiment that I cite:

Twenty tubes were filled each with 0.2 c.c. of 5 per cent suspension of rat corpuscles, 0.05 of dog serum heated to 60° C. for 30 min., and 0.15 c.c. of NaCl solution, incubated at 37° C. for one hour, and divided into two lots of 10 tubes each. The corpuscles in the first tube of each lot were not washed, but the corpuscles in the second tube of each lot were washed once in about 6 c.c. of salt solution; those in the third tube twice, and so on until the 10th tube, the corpuscles in which were washed nine times. The experiment was completed by adding to all the tubes of lot 1, 0.1 c.c. of suspension of dog leucocytes and 0.1 c.c. of NaCl solution, and to all the tubes of lot 2, 0.1 c.c. of leucocytic suspension and 0.005 c.c. of normal serum in 0.1 c.c. of salt solution, incubating for one hour when smears were made, and the percentage of phagocytic mononuclears determined with the following result:

No. of Washings	LOT 1.	LOT 2.
	No Normal Serum	0.005 c.c. Normal Serum
0	10	40
1	10	50
2	5	52
3	0	32
4	0	46
5	0	40
6	2	50
7	0	32
8	0	48
9	2	50
Controls		
NaCl Solution only.....		0
0.005 c.c. Normal Serum in NaCl Solution.....		0

When rat corpuscles are treated with normal and heated dog serum as in the foregoing experiments, there occurs distinct agglutination which often is very marked. The agglutination persists practi-



cally undiminished even after the agglutinated corpuscles are washed 10 successive times in relatively large quantities of salt solution, a new quantity of solution being used for each washing. We have then in this particular instance good examples of firm union of the heat-resistant opsonic element and of the agglutinin with the body upon which they act.

In harmony with the doctrine of tropisms according to which motile organisms orient themselves in response to chemical and other forces, Centanni<sup>1</sup> assumes that the bacterium after being brought into contact with opsonin eliminates a positively chemotropic substance composed of the opsonin and the opsonophile bacterial receptors. According to the theory this chemotropin is the direct agent of phagocytosis. Centanni holds that in suitable mixtures of pneumococci and opsonic serum this soluble chemotropin may form in a few minutes because the phagocytic index obtained with the bacteria of such mixtures is much lower after the bacteria are washed than before. When washed bacteria again come in contact with opsonic serum new chemotropin is given off as shown by the high phagocytic index. Centanni also finds support for this theory in the fact that opsonic serum after being in contact with pneumococci acquire greater chemotactic power than the control serum as tested by means of Pfeiffer's capillary tubes inserted into the subcutaneous tissue, the chemotactic force of the different mixtures being measured by the height and density of the columns of immigrated leucocytes. In connection with this it may be pointed out that the increased chemotactic force may have been owing to other substances than the hypothetical chemotropin and that no matter what the constitution of the chemotropic substance, it may not have been the actual agent of phagocytosis as claimed by Centanni. His cited phagocytic results are in complete harmony with the nature, combining properties and co-operation of the two elements in opsonic serum as set forth in the preceding pages; the formation of the hypothetical chemotropin, however, is directly contradicted by the results of numerous absorption experiments which show that opsonin, at least the specific heat-resistant element, is removed when serum is treated with suitable bacteria or corpuscles.<sup>2</sup>

<sup>1</sup> *Ztschr. f. physiol. Chemie*, 1908, 55, p. 140.

<sup>2</sup> Hektoen, *Jour. Infect. Dis.*, 1908, 5, p. 249; Hata, *Ztschr. f. Hyg.*, 1908, 61, p. 81.



## CONCLUSIONS.

In most cases serum, normal as well as immune, owes its full opsonic effect to a specific thermostable element which unites firmly with the object of its action and to a thermolabile element which seems to remain free in the fluid.

Neufeld's claim that the opsonic action of normal serum in general is caused by the lytic amboceptor and complement and that of immune serum largely by entirely different substances (bacteriotropin and hemotropin) is not established beyond question. On the other hand the view that the opsonic substances in most normal and immune sera are specific and identical, differing only in quantity, harmonizes well with the established facts in regard to these substances as well as in regard to antibodies in general. Hamilton's simple thermostable opsonic substance for certain pseudodiphtheric bacilli answers the requirements of Neufeld's hypothetical bacteriotropins but it occurs, however, in normal as well as immune sera.

In so far as a specific thermostable element is an essential agent of opsonification and phagocytosis the opsonic theory is upheld because it is shown that opsonification is the function of an independent unit in the serum and not of the serum as a whole.

## OPSONINS DISTINCT FROM OTHER ANTIBODIES.\*

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WHETHER the opsonic function of serum and other fluids is dependent on distinct and independent units or on antibodies with other actions as well is an interesting question concerning which there is difference of opinion.

Savtchenko<sup>1</sup> believed that a quantity of fixator in itself insufficient to produce solution of red corpuscles might nevertheless be sufficient to cause their phagocytosis after becoming fixed to them, and, as is well known, Metchnikoff and his followers have held that immune serum may owe its specific powers to substances that stimulate leucocytes directly to phagocytosis at the same time as bacteria or corpuscles that take up fixator are thereby made phagocytatable.

In 1905<sup>2</sup> Dean on finding a thermostable opsonic substance in immune serum assumed on the basis of Ehrlich's theory that a small amount of such substance is present in normal serum; the diminution in opsonic power observed on heating serum, immune as well as normal, was regarded as indicating combined action by two elements, one labile and one stable. Keith<sup>3</sup> urged several considerations against this view and held that if amboceptor-like action is accepted, the existence of special thermostable opsoniferous groups must be admitted in view of the opsonic power of heated serum. In this case the amboceptor would combine the second and third receptor types of Ehrlich.

In a previous article on this subject<sup>4</sup> I concluded that the opsonins in normal and immune serum are distinct from other antibodies because a given serum may be opsonic but not lytic or agglutinating and vice versa, and because in one immune serum results had been obtained that indicated successful separation of the specific opsonic substance from the specific amboceptor.

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<sup>1</sup> *Ann. de l'Inst. Pasteur*, 1902, 16, p. 106.

<sup>2</sup> *Proc. Roy. Soc.*, 1905, 76B., p. 350.

<sup>3</sup> *Ibid.*, 77B., p. 536; *Aberdeen University Studies*, 1906, 21, p. 303.

<sup>4</sup> "Are Opsonins Distinct from Other Antibodies?" *Jour. Infect. Dis.*, 1906, 3, p. 434.

The hypothesis that hemolysis and hemophagocytosis by the same immune serum are owing to different antibodies was brought forward first by Neufeld and Töpfer.<sup>1</sup> In support of this hypothesis Neufeld and Bickel advanced observations to the effect that in the course of immunization hemolytic and hemotropic (opsonic) substances do not appear in the same proportions at the same time, but quite independently of each other; that the two antibodies may be separated both by heat as well as absorption in the cold; that in one case injection with alien blood gave rise to hemolytic and not to hemotropic antibody.

Neufeld's present stand seems to be this: The opsonic effect of normal serum is the result of a slight injury to the bacterium or corpuscle caused by the action of the lytic amboceptor and complement which in this case, however, do not cause lysis; immune opsonins are distinct thermostable substances, of simple nature like agglutinins; the opsonic action of heated normal serum is acknowledged but its cause is regarded as unexplained. The question that at once arises, namely, if amboceptor and complement may cause phagocytosis in the case of normal serum why not also in the case of immune serum, is discussed elsewhere in this *Journal*.<sup>2</sup>

Muir and Martin<sup>3</sup> suggest that immune opsonin may be constituted like agglutinins (because an anticoli serum containing immune body in considerable quantity had no opsonic power) but they are not willing to say that an immune body does not act as opsonin for the reason that they are not familiar with any antiserum with opsonic effect and without immune body. As pointed out in my earlier article on the identity of opsonins, antiserum for streptococci may fulfil this requirement.

Dean's<sup>4</sup> view is that the opsonizing action of serum is due to two substances, "the one thermostable, the substance sensibilisatrice or amboceptor; the other thermolabile, the alexin or complement. The thermostable substance is the essential one, and it may act alone, but its activity is increased by the presence of free complement. The

<sup>1</sup> *Centralbl. f. Bakt., Orig.*, 1905, 38, p. 456. See also Neufeld and Bickel, *Arb. a. d. kais. Gesundh.*, 1907, 27, p. 310.

<sup>2</sup> P. 66.

<sup>3</sup> *Brit. Med. Jour.*, 1907, 2, p. 1409.

<sup>4</sup> *Proc. Roy. Soc.*, 1907, 70B., p. 187.

amboceptor is present in relatively small quantities in normal serum, hence the apparent thermolability of the opsonin in normal serum, whereas in the case of an immune serum the amboceptor is present in a large amount, and perhaps with heightened specific properties, and plays a predominant part, and though heating results in a loss of activity this is only partial. In both cases the loss is due to destruction of complement.” Dean sees difficulty in differentiating between opsonic and lytic substances because the same substance acting in different concentrations may be capable of producing in one case opsonification and in another lysis. In another place<sup>1</sup> he states that a normal amboceptor may be complemented for lytic as well as opsonic action by the same fresh serum.

Wassermann<sup>2</sup> also announces the view that opsonin and bacterial amboceptor are identical and that it depends upon the solubility of the bacteria and on biological relations whether bacteriolysis or phagocytosis shall predominate in a given case.

Certain statements to the effect that normal opsonins are complements, based upon apparent removal of all opsonin by treatment with diverse substances that also remove or neutralize complement, cannot be accepted because of the likelihood that in many such cases the thermolabile activating element only is removed.

In the course of experiments especially in relation to hemolysis and hemophagocytosis I have found that lytic and opsonic properties quite often go together in normal serum, but normal serum may be hemopsonic without being lytic and very often smaller quantities of serum are opsonic than necessary to cause lysis. Of the various sera examined not one that is lytic for the corpuscles in question has failed to reveal some opsonic power at least when using dog leucocytes as phagocytes. The only exception to this statement is frog serum which is markedly lytic but not opsonic for rabbit corpuscles with respect to dog leucocytes. In this case, however, the lysin has the constitution of a typical toxin.<sup>3</sup>

I have also found various examples of persistence of opsonic power of serum after removal or destruction of the lytic amboceptor. The lytic amboceptor may be removed from serum by absorption in the

<sup>1</sup> *Proc. Roy. Soc.*, 1907, 79B., p. 350.

<sup>2</sup> *Deut. med. Wchnschr.*, 1907, 33, p. 1936.

<sup>3</sup> Friedberger, *Centralbl. f. Bakt.*, Orig., 1907, 44, p. 32.

cold and yet the serum may be found to retain opsonic power if carefully tested. An example follows:

Antigoat rabbit serum (heated) 0.025 c.c. + normal guinea-pig serum 0.05 c.c. + 5 per cent goat corp. 1.0 c.c. = Complete Lysis.

Antigoat rabbit serum (heated and exhausted) 0.025 per cent + normal guinea-pig serum 0.05 c.c. + 5 per cent goat corp. 1.0 c.c. No Lysis.

Normal guinea pig serum 0.05 c.c. + 5 per cent goat corp. 1.0 c.c. No Lysis.

The heated and exhausted serum caused, however, marked phagocytosis of goat corpuscles by dog leucocytes.

Again, heating antihuman rabbit serum to 80° C. for 30 min. destroyed all the demonstrable amboceptor for human corpuscles but the opsonin persisted; when 0.025 c.c. of the heated serum was added to 0.2 c.c. 5 per cent suspension of human corpuscles and 0.2 c.c. of suspension of dog leucocytes, well washed, 25 per cent of the leucocytes were found to contain red corpuscles after one hour at 37° C.

Then too a serum may opsonify a certain corpuscle for one kind of leucocyte and not for some other kind. The serum of a rabbit immunized with human blood rendered human corpuscles readily phagocytizable by dog leucocytes, but human leucocytes exercised no phagocytic activity under the same conditions. Human serum does not seem to sensitize for dog leucocytes for which it is markedly toxic.<sup>1</sup> Another example of selective action is furnished by normal rabbit serum which frequently contains an isohemopsonin that renders normal rabbit corpuscles subject to phagocytosis especially by dog leucocytes, rabbit, human, and guinea-pig leucocytes being practically inactive as a rule (Table 1).

TABLE 1.

RABBIT SERUM SENSITIZES RABBIT CORPUSCLES FOR PHAGOCYTOSIS BY DOG LEUCOCYTES BUT NOT BY RABBIT, GUINEA-PIG, OR HUMAN LEUCOCYTES.

Serum	Leucocytes			
	Rabbit	Guinea-pig	Dog	Human
0.1.....	Trace	o	++	o
0.05.....	o	o	++	o
0.025.....	o	o	++	o

Serum (+NaCl when necessary), 5 per cent suspension rabbit corpuscles (washed), suspension of washed leucocytes—equal parts; smears made after 90 min. at 37° C.

On testing the opsonic power of the sera of five rabbits with respect to the corpuscles of these rabbits (serum, 5 per cent suspension of

<sup>1</sup> Goodman, *Jour. Infect. Dis.*, 1908, 5, p. 173

corpuscles, suspension of washed leucocytes [dog] equal parts) the following results were obtained (Table 2):

TABLE 2.  
ISOHEMOPSONIN (AND AUTOHEMOPSONIN) IN SERUM OF RABBITS.

Sera	Corpuscles				
	1	2	3	4	5
1.....	13	7	11	8	10
2.....	5	6	4	6	5
3.....	35	50	35	30	40
4.....	5	6	5	7	9
5.....	9	7	6	8	5

The figures give the percentage of phagocytic cells.

As the table indicates, normal rabbit serum may contain auto-hemopsonin but in a restricted sense in so far as the corpuscles are not rendered as phagocytal for the homologous leucocytes as for dog leucocytes. Each of these five rabbits were now injected intravenously with 2 c.c. of a 10 per cent suspension of washed rabbit corpuscles made up by equal amounts from the blood of the other four rabbits in the set. When the serum of these rabbits was examined nine days later the hemopsonin seemed to be markedly increased in the sera of the injected animals, but I have not been able to obtain any evidence of increase in other animals similarly injected and repeated subcutaneous injections of the animals' own blood corpuscles in increasing quantities have not produced any demonstrable opsonin for rabbit corpuscles. In no case has any isolysin been found.

In goats, however, the amount of isohemopsonin may be distinctly increased on immunization with sheep corpuscles but in no case was there any indication of isolysin in such animals.

Human serum not infrequently contains isohemopsonin (with respect to human leucocytes) but no fixed relation has been found to exist between this isohemopsonin and the isoagglutinins and isolysins.<sup>1</sup>

Dog serum is not destructive of anthrax bacilli because it contains only one of the elements necessary for lysis of the bacilli, namely the amboceptor. Other sera, as, for instance, rabbit serum, contain suitable complement to make the amboceptor in dog serum lytically active. Dog serum, however, is actively opsonic for anthrax bacilli, and this opsonic action is largely lost by heating the serum to 60° C. for 30 minutes but it may be almost fully restored on adding minute quantities of normal dog serum as shown in Table 3.

<sup>1</sup> Hektoen, *Jour. Infect. Dis.*, 1906, p. 721.



TABLE 3.

ACTIVATION OF OPSONIC SUBSTANCE FOR ANTHRAX BACILLI IN HEATED DOG SERUM.

Heated Serum (60°)	Normal Serum	Percentage of Phagocytic Cells
0.1 c.c.	.....	16
.....	0.1 c.c.	80
.....	0.005 c.c.	16
0.1 c.c.	0.005 c.c.	60
.....	NaCl only	10

All mixtures contained 0.2 c.c. of suspension of bacilli and 0.2 c.c. of suspension of washed leucocytes (dog) and sufficient NaCl solution to make the total quantity 0.6 c.c.; incubated for one hour.

At first blush these results speak in favor of the opsonins being distinct from other antibodies. At all events it is established that serum may be strongly opsonic without being lytic or containing lytic amboceptor, so far as is demonstrable with the usual methods. In the case of sera that prove to be lytic but apparently not opsonic the difficulty lies in the possibility that the proper leucocytes may not have been used. On the other side, Dean, Wassermann, and others suggest that failure to obtain lysis may be owing to the state of the object tested and not to the absence of lytic amboceptors. This consideration would apply with most effect to instances in which the bacterium or corpuscle is susceptible to lysis and to opsonification as in the case of anthrax bacilli. Here it could be said that dog complement activates the amboceptor for opsonification and that rabbit complement activates the same amboceptor for lysis. The explanation seems to fall short, however, when applied to bacteria like the streptococcus and pneumococcus that so far as we know are insusceptible of lysis yet readily opsonified. To claim that in this case also lysis is not caused by the opsonic serum on account of the physical state of the cocci would be a pure assumption.

If opsonification and lysis depend upon the same body the lytic and opsonic powers of the serum of an animal in the course of immunization should run parallel. If they do run parallel that fact would not necessarily prove that they depend upon one body, but failure to run parallel would indicate strongly dependence on more than one body. In order to study this particular point as well as to learn something of the general course of opsonin production animals were given single injections of washed alien red corpuscles and the amounts of specific agglutinin, lysin, and opsonin determined at frequent intervals for

some time afterward. The results are shown by means of curves. The figures at the left unless otherwise stated give the highest dilutions of the sera at which the special action in question was present without any doubt and the figures at the top indicate the days. All mixtures contained 0.6 c.c. and in all cases 0.2 c.c. of 5 per cent suspension of corpuscles, the rest being made up by NaCl solution plus serum in the lytic and agglutinating experiments and by NaCl solution plus serum and suspension of dog leucocytes (0.2 c.c.) in the phagocytic experiments. The usual incubation periods were used—two hours for the lytic and one hour for the agglutinating and opsonic mixtures. When the determination was made with fresh serum it was almost always made on the same day as the the serum was drawn and naturally with fresh corpuscles each time. The results of the different determinations consequently are not perhaps quite so strictly comparable as when all were made at the same time with the same corpuscular suspension, the serum from the various bleedings having been kept constantly at 0° C. In the latter case the sera were first heated to 60° C. for 30 min. and proper quantities of a suitable fresh serum used as complement for the determination of lysis. The two methods appear to yield results that are much alike. On account of certain peculiar changes to which rat corpuscles are prone when washed and suspended in salt solution, fresh suspension should be used invariably.

The charts show that in most instances the agglutinin, lysin, and opsonin curves run parallel and present the normal type of antibody curve. This is especially noteworthy in the case of the agglutinin and opsonin curves. That agglutinins and opsonins are distinct substances is indicated in several ways but most strongly perhaps by the facts that the action of agglutinins is not materially reduced, if at all, by heating serum to 60° C. for 30 minutes, and that agglutination by heated serum is not definitely promoted by small quantities of fresh serum as in the case of opsonification by heated serum. In some instances the increase above normal in the agglutinin and opsonin is disproportionately greater than that in the lysin. This is notably so in dogs after the injection of rat corpuscles. In dogs splenectomy may interfere markedly with the production of antibodies after injection of rat corpuscles but not always. In such instances

careful post-mortem examination has not revealed any accessory spleens that might have assumed whatever part the spleen may take in the production of antibodies. In the two splenectomized dogs represented on Chart 2 the lysin did not rise above normal, while the agglutinin and opsonin gave a very decided rise indeed. The indication is then, at least in this case, that the lysin is distinct from the opsonin and the agglutinin.

## CHARTS

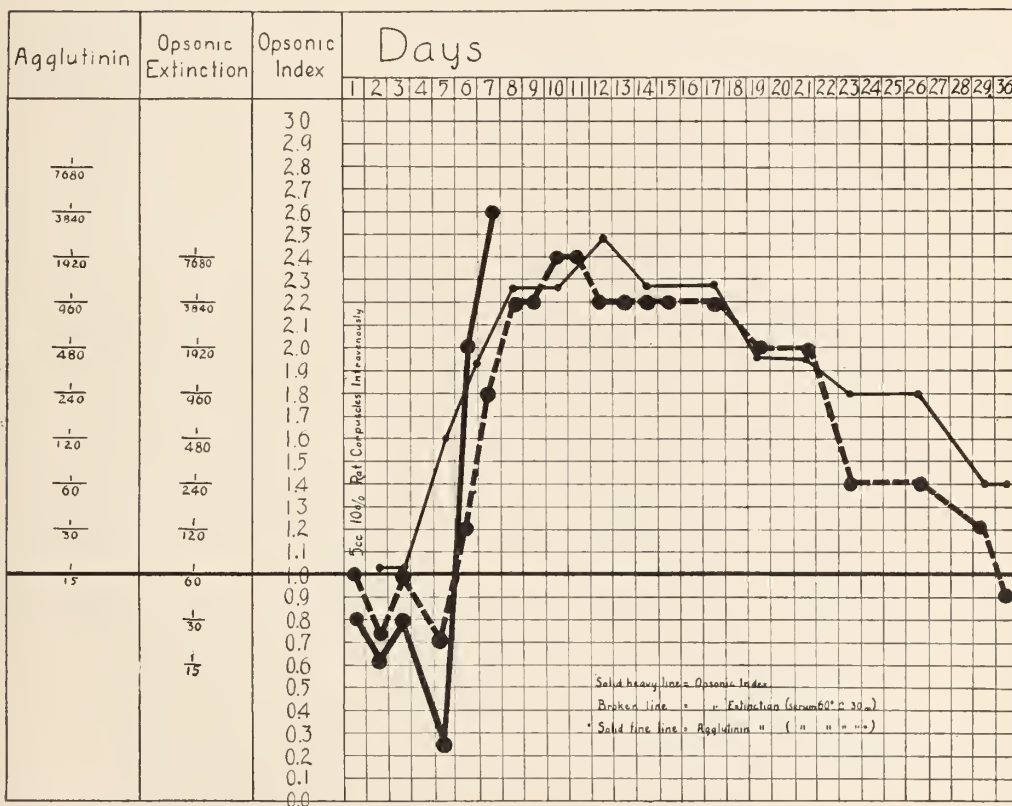
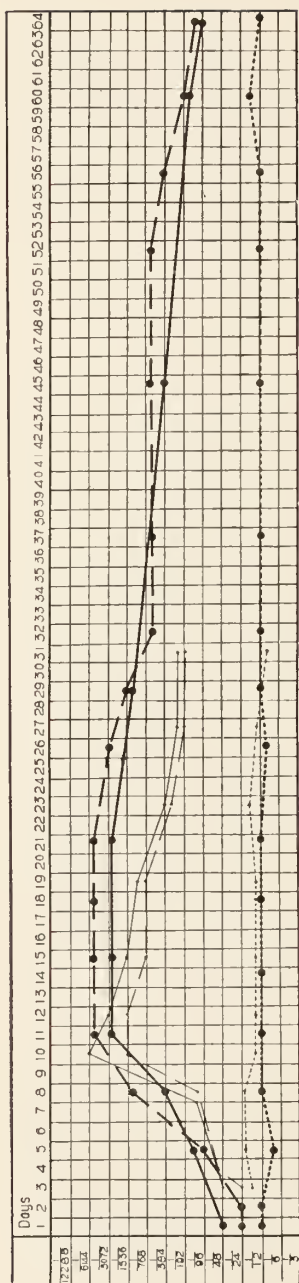


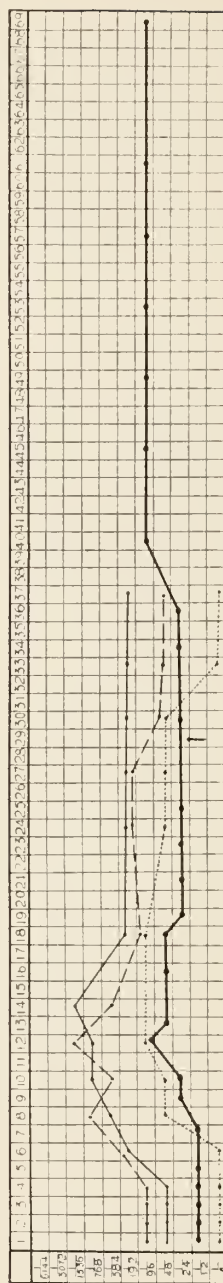
CHART 1.—Antibodies in serum of dog injected with rat corpuscles. (Opsonic index estimated by comparison of number of phagocytic mononuclear cells in mixtures with fresh serum of injected dog and in mixtures with normal dog serum under conditions of absolute comparability. Opsonic extinction of normal serum heated to 60° C. 30 m. in 60, agglutinin extinction 15.)



Heavy lines—5 c.c. 10 per cent susp. rat corpuscles intravenously and splenectomy on second day. Heated serum. Complement—0.02 guinea-pig serum.  
Fine lines—5 c.c. 10 per cent rat corpuscles intravenously on first day and splenectomy on fifth day. Fresh serum.

Solid lines—Opsonin; Broken lines—Agglutinin; Dotted lines—Lysin.

CHART 2.—Antibodies in serum of splenectomized dogs after injection of rat corpuscles.



Fine solid line—Opsonin; Fine broken line—Agglutinin; Dotted line—Lysin. In serum of dog splenectomized on 4th day after injection of rat corpuscles. Heated serum. Complement. 0.013 guinea-pig serum.

Heavy line—Agglutinin and Opsonin in serum of dog injected with rat corpuscles; splenectomized on previous day. Reinjecting on the 20th day.

CHART 3.—Antibodies in serum of splenectomized dogs after injection of rat corpuscles.

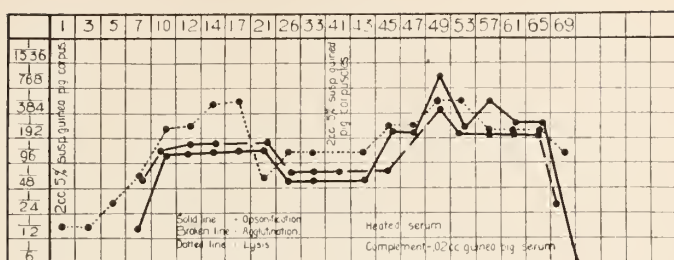


CHART 4.—Antibodies in serum of rabbit after injection of guinea-pig corpuscles.

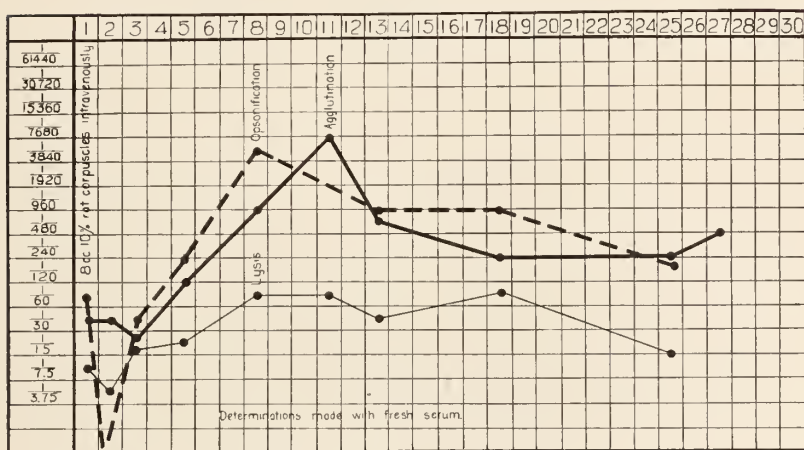


CHART 5.—Antibodies in serum of dog injected with rat corpuscles.

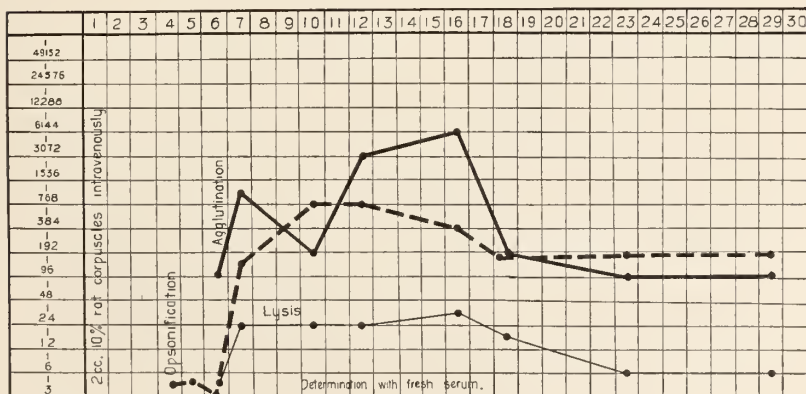
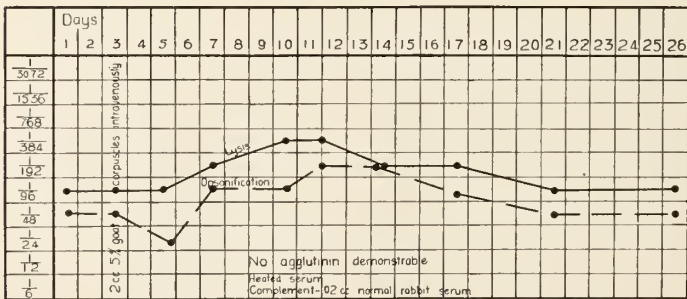
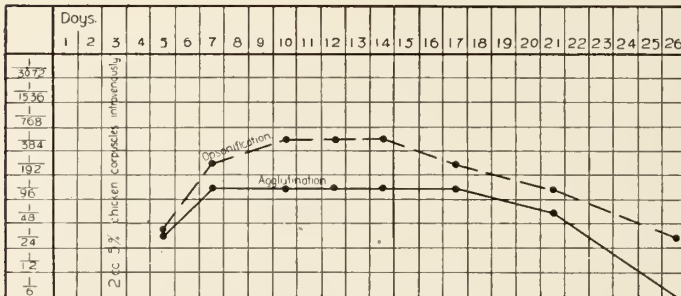
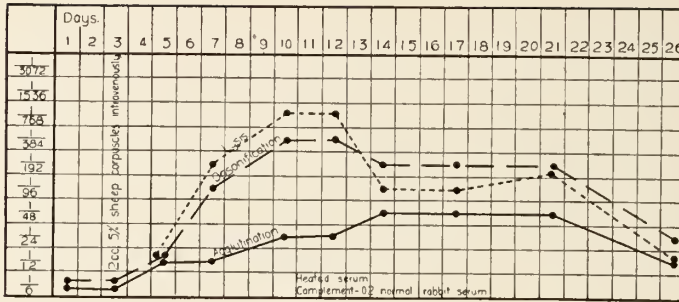


CHART 6.—Antibodies in serum of rabbit injected with rat corpuscles (no action of any kind in dilutions of 1 to 3 until fourth day).



CHARTS 7, 8, 9.—Antibodies in sera of rabbits injected with goat, sheep, and chicken corpuscles respectively.



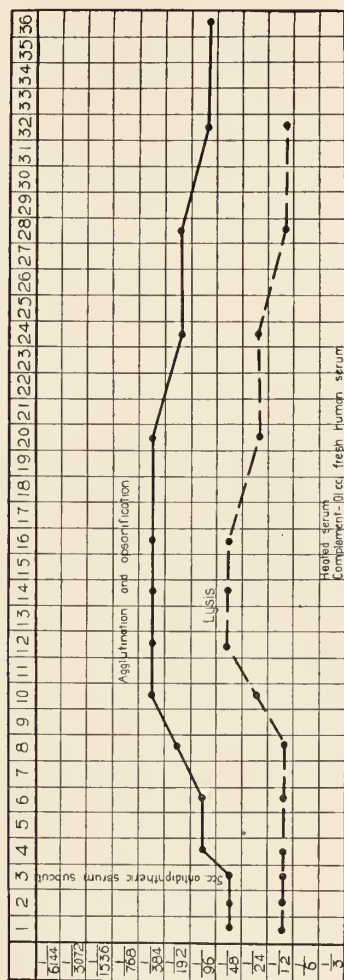


CHART 10.—Antibodies for horse corpuscles in serum of man after injection of antiphthoric serum (mild form of serum disease, second day).

## A CASE OF NON-INHERITANCE OF FLUCTUATING VARIATIONS AMONG BACTERIA.\*

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THE subject of variation among the bacteria is of obvious importance to those who are concerned with the study of these organisms in their relations to everyday life. The pathologist and the sanitarian and the soil bacteriologist are vitally concerned with the question as to how far the types with which they work are constant and invariable, and to what extent they may modify and interchange their characters. The problem has a much wider significance, however. The bacteria exhibit protoplasm in one of its simplest forms. The absence of amphimixis and the extent to which these organisms are exposed to the direct influence of the environment make it possible to study in them some of the fundamental problems of heredity and variation in their simplest terms. The rapidity with which bacteria multiply and the ease with which they are cultivated make it reasonable to prophesy that the study of bacterial variations will some day throw important light on such central problems of general biology as the origin of species.

There are two or three fundamental types of variations among the bacteria which ought to be distinguished as clearly as possible. In the first place many of the observed differences in bacterial cultures are not variations at all but temporary modifications of behavior due to the direct effect of existing conditions of cultivation. Pigment production at 20° but not at 37° is a gross and obvious example; but many properties described as characteristic are really of the same sort. The shape and structure of colonies on gelatin are, for example, largely the result of the density of the medium and the moisture of the atmosphere. Of true protoplasmic variations, which lead to characteristic differences in behavior under identical conditions, there must be again two distinct types. The first sort are due to causes operating within the cells, variations which arise between the offspring of one original bacterium when growing under approximately the same con-

\* Received for publication January 6, 1909.

ditions. The origin of these variations, in the absence of amphimixis, must apparently be sought in inequalities of division between individual bacterial cells in the process of reproduction. Variations of this general type may be minute fluctuating variations or well-marked, sharply separated sports, or mutations. Fluctuating variations are common and mutations have been recorded by a few observers (Neumann,<sup>1</sup> Beyerinck,<sup>2</sup> Neisser,<sup>3</sup> Massini<sup>4</sup>).

A second type of variations, distinct from these intrinsic ones, is due to the effect of special environmental conditions upon a succession of bacterial generations. These have been called impressed variations, among the bacteria. Impressed variations may be due to a direct reaction between the bacterial protoplasm and its environment as is probably the case in the exaltation of virulence by successive animal inoculations; or they may be due to a selective force acting upon fluctuating variations of the type earlier discussed. Which of these two latter sorts of variation we are dealing with in a given case it is often difficult to say.

In a recent very valuable contribution to this subject, Goodman<sup>5</sup> has shown that, by a gradual process of selection of impressed variations, organisms of the *B. diphtheriae* group could be profoundly modified in regard to acid production. A culture of *B. diphtheriae* which produced an acidity of about 2.0 per cent normal in dextrose broth was inoculated into 15 tubes of dextrose broth and the final acidity determined by titration. From the tubes showing the maximum and minimum acidities, two sets of 15 tubes each were inoculated, and designated the high and the low series, respectively. Each series was carried on for 36 transfers, the tube of maximum acidity in the high series and the tube of minimum acidity in the low series being used in each case for the next transfer. At first the high and low series did not differ widely; but after 15 transfers they began to diverge. The final acidity of the high series was nearly 5.0 per cent normal while the low series finally produced an alkaline reaction.

In this important investigation it was demonstrated that a marked

<sup>1</sup> *Archiv f. Hyg.*, 1897, 30, p. 1.

<sup>2</sup> *Kon. Akad. v. Wetenschappen*, Amsterdam, 1900.

<sup>3</sup> *Centralbl. f. Bakt.*, 1906, 38, Abth. 1, Ref. Beiheft, p. 98.

<sup>4</sup> *Archiv f. Hyg.*, 1907, 41, p. 250.

<sup>5</sup> *Jour. Infect. Dis.*, 1908, 5, p. 421.

change in a fundamental bacterial character could be brought about by a process of selection. Peckham<sup>1</sup> and Horrocks<sup>2</sup> similarly report that the indol reaction and other properties of the *B. coli* group may be modified by cultivation under favorable conditions. Twort<sup>3</sup> has more recently been able to develop fermentative power in bacteria of the *B. enteritidis* type by continued cultivation in sugar media. In all these cases the modification produced is in the nature of an impressed variation, since it is the result of prolonged cultivation under special conditions. In Goodman's experiments it seems probable that the chief force at work was the progressive selection of spontaneous variations in bacterial activity. An important part may also have been played, however, by the direct influence of the environment. The organisms of his high series were exposed for transfer after transfer to high acidities and those of the low strain to low acidities. Under such circumstances there must have been a tendency to acquire or to lose a tolerance of the acid reaction as a result of the direct action of the environment.

The present investigation was planned so as to exclude the latter factor, the direct effect of the environment, and to deal with the inheritance of spontaneous variations of the fluctuating type. Meyer<sup>4</sup> and Lepeschkin<sup>5</sup> have recorded a tendency to the inheritance of branching in the offspring of certain cells of *B. cohaerans* and *B. Berestnewi*; and a very beautiful piece of work on this subject has recently been published by Barber.<sup>6</sup> He found that elongated cells occurred rarely but with considerable regularity in cultures of *B. coli* and that by isolating these cells and breeding from them he could get a new race, constant to the elongated form and showing other characteristic differences from the parent strain. Similar, though less conclusive, results were obtained with *B. typhi*, and in one case an apparently non-spore-forming race was derived from *B. megatherium*. Barber was evidently dealing with mutations entirely comparable with those which appear in the evening primrose; and the branching cells studied by Meyer and Lepeschkin were also apparently discontinuous variations. In regard to fluctuating, continuous

<sup>1</sup> Jour. Exper. Med., 1897, 2, p. 549.

<sup>2</sup> Jour. Roy. Army Med. Corps, 1903, 1, p. 362.

<sup>3</sup> Proc. Royal Soc., Series B., 1907, 79, p. 329.

<sup>4</sup> Centralbl. j. Bakt., 1901, 30, Abth. 1, p. 49.

<sup>5</sup> Ibid., 1904, 12, Abth. 2, p. 641.

<sup>6</sup> Kansas Univ. Sci. Bull., 1907, 4, p. 1.

variations Ružicka,<sup>1</sup> Conn,<sup>2</sup> Smith,<sup>3</sup> and Sullivan<sup>4</sup> have reported significant changes due to selection but the variations (liquefying power, or chromogenesis) were not measured by exact quantitative methods.

The organisms chosen by us for study were strains of the two types A and B of the paratyphoid bacillus of Schottmüller. These types are alike in fermenting dextrose with the production of gas and acid, in slightly acidifying lactose media without gas formation, and in failure to form indol in peptone media. They differ mainly in their action on milk and in the vigor of their growth on media. Type A grows like the typhoid bacillus on gelatin and potato and gives a slight but permanent acid reaction in milk. Type B is more vigorous, forming a richer growth on gelatin and potato, and in milk producing first a faintly acid reaction and after four or five days an alkaline one. Type B also produces a slightly higher acidity and a larger amount of gas in dextrose broth. The property of acid formation in dextrose broth was selected for study, on account of the ease and precision with which it can be measured.

Cultures of the two types of the paratyphoid bacillus were obtained through the courtesy of Mr. B. R. Rickards, from the laboratory of the Boston Board of Health, where both had originally been isolated from cases of paratyphoid fever. Their reactions were re-examined and found to be entirely characteristic. Each culture was plated out on a series of gelatin plates, so as to get well-isolated colonies and 100 agar tubes were inoculated from 100 separate colonies of each strain. These tubes were incubated at 20° until a distinct growth appeared, and a tube of 1 per cent dextrose broth was inoculated from each. The dextrose broth tubes were called Series I (Type A), and Series IV (Type B). After 72 hours at 20° the acidity in each of the 200 tubes was determined by titration in the cold against twentieth-normal sodium hydroxid, using phenolphthalein as an indicator. Sterile tubes of the same batch of broth were titrated at the same time and their average acidity subtracted from that of the inoculated tubes.

<sup>1</sup> *Archiv f. Hyg.*, 1899, 34, p. 140.

<sup>2</sup> *Jour. Boston Soc. Med. Sci.*, 1900, 4, p. 170.

<sup>3</sup> *Ibid.*, 1900, 4, p. 95.

<sup>4</sup> *Jour. Med. Res.*, 1905, 14, p. 109.

The results are shown in Table I, Columns I and IV, the figures opposite each acidity-class indicating the percentage of the 100

TABLE I.  
DISTRIBUTION OF PARATYPHOID CULTURES ACCORDING TO ACID PRODUCTION.  
(Percentage of Cultures in Each Class.)

ACIDITY-CLASS Per cent Normal	TYPE A			TYPE B		
	I	II	III	IV	V	VI
I. 15-1.20.....	2	1	*			
I. 20-1.25.....	3	6	1		1	
I. 25-1.30.....	11	8	7			
I. 30-1.35.....	8	18	15			
I. 35-1.40.....	13	11	14	3	1	*
I. 40-1.45.....	24	26	28	2	17	1
I. 45-1.50.....	22	16	18	2	11	10
I. 50-1.55.....	8	6	8	7	22	8
I. 55-1.60.....	7	6	8	14	13	22
I. 60-1.65.....	2	2*	1	21	14	12
I. 65-1.70.....				26	9	24
I. 70-1.75.....				11	7	11
I. 75-1.80.....				13	4	4
I. 80-1.85.....				1	*	4
I. 85-1.90.....					1	
I. 90-1.95.....						4
Mean.....	1.41	1.40	1.42	1.64	1.55	1.63
Median.....	1.43	1.41	1.42	1.67	1.54	1.64
Standard deviation	0.49	0.50	0.43	0.50	0.59	0.57

cultures falling in that class. The same results are shown graphically in the upper section of the figure, plotted as polygons of frequency. The abscissae are acidity-classes and the ordinates indicate the percentage of cultures in each class.

The acid production for each type forms a normal curve of frequency with a mean for Type A at 1.41 and for Type B at 1.64. The median is close to the mean in each case and the Standard Deviations are respectively 0.49 and 0.50. The range for Type A is from 1.15 to 1.65; and for Type B from 1.35 to 1.85. Evidently the two types, while they overlap, vary about distinct centers.

The differences between the individual members of each set of 100 tubes may have been due in part to variations arising from some cause in the broth tubes themselves. If, however, there were any intrinsic differences in dextrose-fermenting power between the 100 single bacterial cells which formed the starting-point for each series, a part of the observed variation in acidity must have been due to these initial differences. A control experiment with 50 dextrose tubes inoculated from the same agar streak showed much less variation in acidity than was apparent in our main experiment. In this special



test (with Type B), 60 per cent of the acidities fell between 1.55 and 1.65. The wider variation in the series inoculated with strains derived from separate colonies may therefore be attributed to

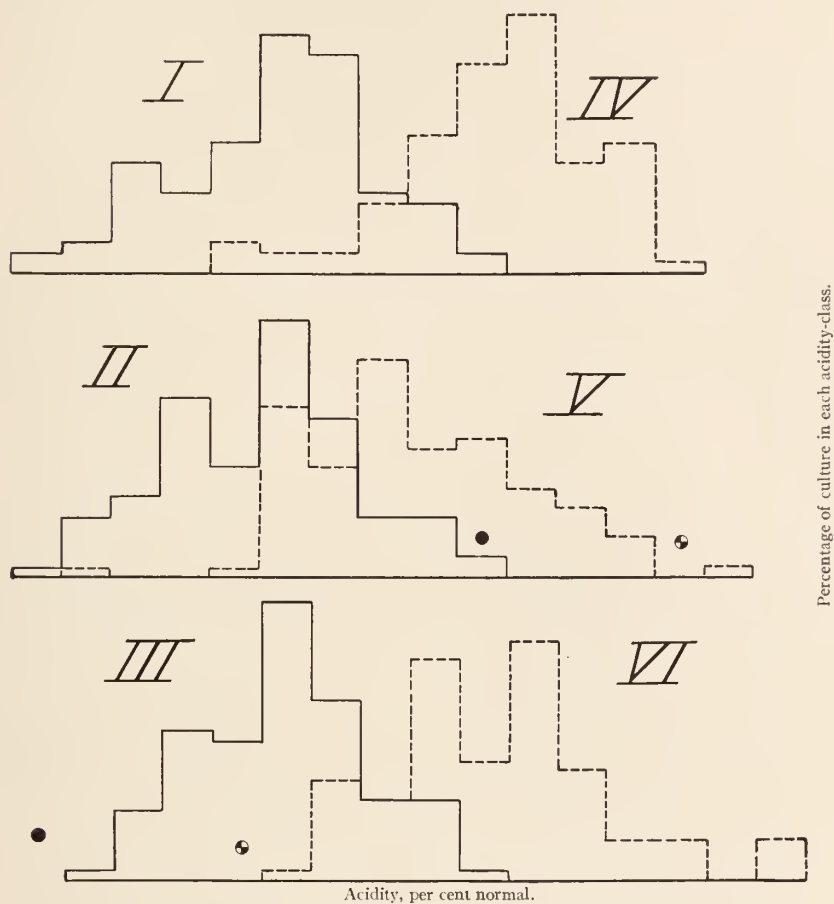


CHART I.—Distribution of Paratyphoid Cultures According to Acid Production.

— Type A      --- Type B  
 ● Parent strains for Type A  
 ⊕ Parent strains for Type B

intrinsic differences between the parent cells of the colonies themselves. It was the inheritability of these original variations that we wished to examine. We did not therefore make further inoculations from the broth cultures themselves, in which the organisms had been

actually exposed to varying degrees of acidity. We went back to the original agar streaks and selected, first the two streaks which, when inoculated into broth, had shown the highest acidities for their respective types. These streaks were plated out and from colonies on the plates new agar streaks were made, Series II and V. The cultures in Series II were derived from the parent streak of the highest acidity in Series I, Type A (acidity between 1.60 and 1.65). Series V was derived from the parent streak of the highest acidity in Series IV, Type B (between 1.80 and 1.85). From each of these series dextrose broth tubes were inoculated and titrated as before; and the results are indicated in Columns II and V of the table and in the middle section of the figure. The whole procedure was then repeated in Series III and VI with cultures made from individual colonies derived from the parent streaks of the tubes which showed minimum acidities in Series I and IV. One of the streaks in Series I, Type A, which gave in broth an acidity between 1.15 and 1.20 was used as the starting-point for Series III; one of the streaks in Series IV, Type B, which gave acidities between 1.35 and 1.40 was the starting-point of Series VI. The source of each series, as regards acid-producing power, is indicated on the table and in the figure by a star or circle. In Series II, III, V, and VI the actual numbers of subcultures examined were respectively 88, 98, 72, and 74, instead of 100; but all figures are reduced to percentages so as to be comparable.

It is at once evident from the table and from the figure that there was no apparent modification of the character of either type as a result of selected fluctuating variations. Each curve reverted completely to the original mean of its type. Thus Series II, derived from a streak the direct inoculation of which into broth produced an acidity between 1.60 and 1.65, had its mean, not at 1.60 but at 1.40; conversely Series VI, derived from a streak which originally produced an acidity between 1.35 and 1.40, had its mean, not at 1.40 but at 1.63. The only irregularity in the whole experiment is in Series V. This series was derived from the parent streak of a tube showing high acidity; but, instead of having a high mean, it showed a mean of 1.55, below that normal for the type, with a high standard deviation. This aberration was undoubtedly due to the fact that the incubator in which this particular series was cultivated in broth was cooled below

room temperature, through an oversight in regard to the regulating apparatus. Altogether it is evident that if there were any protoplasmic differences in acid-producing power between the original cells from which the individuals in Series I and IV were derived they were only transient; and in their later descendants there was a complete reversion to the general mean of the parent strain. Taking into consideration the close relationship of Types A and B and their very slight initial differences, the constancy with which these differences were maintained is a striking example of the possible fixity of bacterial types in the absence of modifying environmental conditions.

The difference between our results and those reported by Goodman may be due to several causes. His cultures were carried through a great many transfers, so that slight initial differences were accumulated. On the other hand, the mean of 100 cultures is a fairly delicate measure and ought to detect quite minute variations of character. His cultures were exposed to the direct modifying effect of media of greater or less acidity; and this factor may be of importance. It scarcely seems likely, however, that Goodman's results could have been attained without the existence of intrinsic variations for selection to work upon. A preliminary study, in which he determined the acid-production of 103 strains of bacilli of the diphtheria group, showed indeed a variation in dextrose broth from a reaction of 0.1 per cent normal to a reaction of 4.0 per cent normal. The curve plotted from his results was a very low and irregular one with a single mean, mode and median, all between 1.75 and 1.80, and a standard deviation which we have calculated as about 0.95. If all our 538 cultures of both paratyphoid types were grouped in a single array they would fall between the limits of 1.15 and 1.95; yet in this narrow range two distinct modes would be apparent, one between 1.40 and 1.45, and the other between 1.55 and 1.60, corresponding to the two types which we have found to be apparently distinct. It seems evident from all these facts that the property of acid-production is relatively variable in the *B. diphtheriae* group and relatively stable in the paratyphoid group.

A STUDY ON THE LIFE HISTORY OF A FLAGELLATE  
(*Crithidia melophagi*, n. sp.) IN THE ALIMENTARY TRACT  
OF THE SHEEP-TICK (*Melophagus ovinus*).\*†

LEROY D. SWINGLE.

SINCE the year 1902 insect flagellates have been a subject of much investigation in view of the possibility of their being developmental stages in the life cycle of the hemoflagellates. In a paper by Novy, MacNeal, and Torrey (1907), a most excellent résumé of the present knowledge concerning these parasites is given. It would therefore be entirely superfluous to review the literature in this connection.

This flagellate, to which I have given the name *Crithidia melophagi*, has been studied to some extent by E. Pfeiffer (1905). He claims the discovery of it for L. Pfeiffer about ten years before. As far as I am aware, no one else has given attention to it, though Dr. B. H. Ransom called to my notice the similarity between these forms and a flagellate which I (1907) discovered in the rat flea (*Ceratophyllus fasciatus*), about which more will be said later.

The ticks were removed from the sheep and examined, some immediately and others at various times after removal, so as to discover all possible stages and conditions of the parasites. To remove the stomach the posterior end of the abdomen was clipped with small scissors, and then, holding the tick on a slide by grasping the thorax with a pair of forceps, the contents of the abdomen were pressed out into a drop of salt solution placed on the slide. The pressure was effected by placing a needle on the anterior part of the abdomen and then drawing it toward the posterior end pushing the abdominal contents before it. The whole viscera including the oesophagus and rectum were thus squeezed out. With dissecting needles the digestive tract with its appendages was separated from the other viscera and straightened out, when it measured over an inch in length. This can all be done with the naked eye and without rupturing any portion of the tract. Sometimes the stomach wall is sufficiently clear for the moving flagellates within its lumen to be seen. If the ticks have not been long removed from the sheep, the anterior third of the stomach will be full of fresh blood. The posterior two-thirds contains blood in all stages of digestion, the more posterior parts being darker and often almost black. In starved ticks the anterior third is empty, while the rest contains the dark disintegrated blood. The posterior portion is often so filled with parasites that there is very little space left for food material.

For sectioning, the tract was put into a fixing fluid, Hermann's, Merkel's, or Zenker's. Having straightened it out it was sectioned its whole length serially, and thereby the exact conditions of the parasites in all parts of the tract were determined. In other

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† Studies from the Zoölogical Laboratory, The University of Nebraska, under the direction of Henry B. Ward, No. 91.

cases the stomach contents were teased in a drop of salt solution on a slide. By cutting the stomach into pieces and transferring these to other slides the flagellates from various regions were separated. They were either studied in the living condition or were used for permanent mounts. In making the stained preparations a small drop of the salt solution containing the parasites was placed on a slide and, before it became entirely dry, some killing fluid was dropped on, which fixed them to the slide. For this purpose various killers such as methyl alcohol, absolute alcohol and ether, Merkel's, Carnoy's, and Zenker's fluids were used. A hot solution of corrosive sublimate and 95 per cent alcohol, made by mixing two parts of 95 per cent alcohol with one part of a saturated aqueous solution of sublimate, was also used. While very good results were obtained by this method, perhaps the most satisfactory one is to invert the slides for ten minutes over the mouth of a jar containing formol, then allow them to dry. As the salt solution dries down the salt crystallizes so that the parasites are obscured. To remove the crystals the slides were then washed in hot water and again left to dry. Results obtained by fixing with heat or from allowing the preparations to dry before fixing the flagellates, as others have done, were always inferior to those obtained by the above methods. I tried also teasing the stomach contents in fluid from the body cavity and then smearing the mixture as in making blood films. A serious objection to this method is that some of the parasites are distorted and one cannot be sure whether he is studying a normal or a distorted form. This goes to confirm MacNeal's opinion (1904) and my own conviction that blood trypanosomes are often badly distorted in smears. The trypanosomes, I believe, suffer even more disaster from such treatment than these flagellates, because they are not so firm and rigid as the latter. The contact preparations were stained according to Wright, Giemsa, or with iron hematoxylin. Mayer's hemalum and iron hematoxylin were used for staining sections. As mounting media, balsam, damar, and cedar oil were used. The Zeiss apochromatic outfit, including the 2 mm. and 3 mm. oil immersion lenses, and the apochromatic condenser was used both with daylight and the Welsbach gas burner.

I follow Woodcock (1906) and Minchin (1908) in using trophonucleus for nucleus and kintonucleus for blepharoplast.

These flagellates present as much variability in size and shape as the rat trypanosome during the period of multiplication. They vary from a very long slender form down to a small spindle-shaped flagellate or spherical mass. Thus, no general description will suffice but it is necessary to describe each form separately.

The long forms, flagellum included, measure about 30 to 45  $\mu$  long by 2  $\mu$  wide. Sometimes, however, they reach a length of 60  $\mu$ . On the other hand, the young dividing forms may be only 12  $\mu$  in length. The typical forms have the appearance of a thin band thickened and widened in the middle. They are often spirally twisted, sometimes to the extent of three or four turns. A well-developed undulating membrane is present (Plate 3, Figs. 1-4). It is formed by the extension of the anterior end of the body along the flagellum,



generally to a point within 2 to 4  $\mu$  from its end. In some cases it extends clear to the end. The membrane is very thin near the end, while farther back it becomes thicker, especially along the side opposite the bordering flagellum. The flagellum does not pass into the body but proceeds along its thin membranous edge to the kinetonucleus which lies against the wall (Figs. 2, 3, and 6). That such is the case was plainly shown by staining a long time with iron hematoxylin and then destaining thoroughly, so that the flagellum stood out as a black line. The parasites not being smeared on the slide stood out so that their true form could be determined. Since the membrane is formed by the end and side of the body, there is no line of separation between the body and the membrane. The whole anterior end of the flagellate nearly back to the kinetonucleus takes part in the undulating movements. The posterior half of the body is stiff and firm. This rigidity is not due to a central axis such as described by Prowazek (1904) for his forms, nor to a thick wall. The wall is so thin that it can be demonstrated with no certainty neither in stained preparations nor by crushing the flagellates in the living state. The rigidity seems rather to be a property of the cytoplasm itself. Prowazek ascribes the cause for rigidity in his forms to this central axis which is nothing more than a couple of spirally twisted threads running longitudinally through the body. According to the laws of mechanics, it is impossible to say that the rigidity is due to such a structure. It would have been far more reasonable and plausible for him to have attributed the cause of rigidity to the cuticle (periplast) which, according to him, is so firm that it remains as a delicate sheath when the endoplasm is forced out by pressure.

There is but one flagellum present. Stained according to Giemsa or Wright this point was at times doubtful. But in the forms stained a long time with iron hematoxylin there was never any doubt, the flagellum standing out as a black line. Then, too, occasionally flagellates were found with the flagellum stripped from the membrane back nearly as far as the kinetonucleus. In these forms never more than one flagellum could be seen. This condition was described by Prowazek for his form, but he states that there are two flagella with a membrane stretched between them. He used Giemsa's stain, which gave doubtful results in my hands.



The trophonucleus is situated about the center or in the posterior third of the body. It is round or elliptical and varies in size. On the average it measures about  $1.7\ \mu$  by  $2.5\ \mu$ . When stained with the hematoxylin a central deeply staining granule is plainly visible. This was seldom seen when Giemsa's or Wright's stain was used. It is exceedingly brilliant when stained with borax carmine. The chromatin is in the form of a reticulum upon which a number of granules is to be found. They cannot be differentiated with the hematoxylin but are distinct when stained according to Giemsa. They vary in number from four to 16. The most frequent number is from seven to 11. When the number is small they are all arranged in the circumference of the trophonucleus, but when the number is large some occupy a more central position. Especially in the latter case, the trophonucleus is very similar to nuclei of multicellular animals. Since these granules occur in varying numbers and in resting nuclei, they should not be considered as chromosomes. I hold that they are comparable to the net-knots of false nucleoli in nuclei of higher forms. The central granule has a stronger affinity for borax carmine and the hematoxylin than does the other nuclear material. I have never been able to distinguish these structures in divisional stages, the nucleus appearing as a dense structureless mass.

The kinetonucleus lies in a transverse position anterior to or at the side of the trophonucleus. In profile view it is seen to lie against the edge of the body on the same side as the flagellum. Stained with iron hematoxylin or safranin it appears either as a straight rod with square or rounded ends or like two balls which have partially fused. But when stained according to Giemsa it often appears in addition to the above forms as an ellipse or as a crescent with the horns pointing anteriorly. This crescentic appearance is sometimes due to the fact that the posterior rim of the ellipse is heavily stained while the rest of the ellipse is faintly stained or not at all. The kinetonucleus is larger when stained according to Giemsa than when other stains are used. It appears from this fact that there is an outer area which stains only with Giemsa. At times it is nearly as large as the trophonucleus and presents much the same structure. It may or may not lie in a clear area. The flagellum at its juncture with the kinetonucleus often remains unstained, as in the trypanosomes. The cytoplasm

is clear, finely granular, and reticulate. Generally small vacuoles, appearing in the living condition as refractive spots, are to be found mostly posterior to the trophonucleus. Between the vacuoles from four to 12 round or irregular granules, which stain with Giesma like the kintonucleus, are present. They are deeply stained even when the trophonucleus is barely visible (Fig. 2). Iron hematoxylin, however, does not differentiate them. If they are chromatin it is strange that they are not differentiated at all with iron hematoxylin, but with Giesma stain even more readily than the trophonucleus. There are, however, other masses (probably chromatin) sometimes present that do stain with iron hematoxylin. No posterior diplosome could be found.

The posterior end may be abruptly pointed or bluntly rounded but seldom drawn out into a long tapering point (Figs. 1-4). Instead, it is often rounded off so as to form a large knob containing the trophonucleus or kintonucleus, or both (Figs. 7, 8). In these cases the anterior end remains about the same as before. Others swell out in the region of the trophonucleus to form a ball-like structure in the middle of the body. Both of these types are very commonly found. Stiff spindle-shaped forms are also abundant (Fig. 5).

In sections these forms are found free in the lumen or attached in a single layer to the wall of the stomach by the anterior end. There are always as many attached as can possibly find room, no matter what the food condition may be. The remaining individuals are either free or are attached to free masses of epithelium. Sections of the malpighian tubes showed both long and short flagellate forms, some of which had traveled a considerable distance from the juncture of the tubes and the stomach.

The very long forms were never seen in process of division. In several cases, however, two trophonuclei were found. The long forms are to be regarded as adult or senile forms, which have originated by growth from the smaller types. This idea is confirmed by the fact that they are the first to die when the contents of the stomach are kept in salt solution. Some of the smaller forms remained alive more than five days at room temperature. Division, which is longitudinal, regularly takes place in the smaller forms (Plate 4, Fig. 9). This one figured is an exceptionally large divisional form. The

kinetoculus divides first and a new flagellum arises apparently by splitting off from the old one. The trophoculus then divides and later the cytoplasm constricts. In no case was there any indication of mitosis. It is a striking fact that in spite of the enormous number of parasites present, division stages are very rare. However, in one tick gorged with dark-colored blood division forms were exceptionally abundant. Although they are scarce, some division stages are always present.

One is surprised at the vast number of parasites to be found by examining the stomach contents in the living condition after teasing the digestive tract in salt solution. They fill the digestive tube so full in the posterior two-thirds that it is a wonder how any food could pass through without crowding them out. Large rosettes with flagella centrally directed are present. It is a common thing to see two long slender forms agglutinate alongside each other with the flagella pointing in the same direction. Occasionally several are massed together in such fashion. There is no fixed law, however, causing them to agglutinate with flagella centrally directed, although this method is certainly the typical one. Two flagellates may, instead of coming together side by side with the flagella pointing in the same direction, agglutinate so that the flagella point in opposite directions. In this case the parasites tend to move in opposite directions, the flagellate end being the anterior end. The result is that they often slip past each other, so that only the posterior tips are in contact. Very frequently such couplets are seen, the stronger of them pulling the other through the fluid. Novy, MacNeal, and Torrey say that in agglutination rosettes the posterior ends are central in position as in the trypanosomes, but in division rosettes the flagella are centrally located as in the division rosettes of trypanosomes in culture. The former statement does not hold true in these forms. Whether the rosettes are formed by agglutination or division, they regularly have the flagella centrally directed.

In the intestine, which begins at the juncture of the malpighian tubes with the stomach, forms peculiar to this region were found. They correspond to the "formes grégariennes" of *Herpetomonas subulata* as described by Léger (1904). Just posterior to the valve, which is at the point where the malpighian tubes enter the digestive

tract, the intestine is much larger than the end of the stomach just anterior to the valve. It is funnel-shaped, the bowl being directed anteriorly. Lining the posterior wall of the valve which projects a short distance posteriorly into the funnel are several layers of parasites (Fig. 14). These forms are large, always rounded off at the posterior end. Those in the outside layer often measure as much as  $20\ \mu$  in length. The individuals in the deeper layers are smaller. On the sides of this dilated portion of the intestine the parasites are smaller, yet very similar. Generally they are arranged in several layers, but there may be in some places only one layer. The kinetonuclei of the individuals in the deepest layer are anterior to the trophonuclei, while they are clear in the posterior end of the parasites of the superficial layer. The parasites of the intermediate layers have the kinetonuclei at the side of the trophonucleus (Fig. 18). These forms measure 2 to  $3\ \mu$  wide and about 4 to  $6\ \mu$  long, including the free flagellum when present. Passing posteriorly the intestine quickly narrows to a much smaller caliber where the parasites become smaller, measuring only  $3\ \mu$  in length. In the rectum either these small pear-shaped forms or small spherical forms are found lining the wall in layers. Throughout the lumen of the intestine and rectum free forms (Fig. 16) and rosettes (Fig. 17) with flagella centrally directed are present. These pear-shaped forms or "gregarine" type are either pointed or truncated and the flagellum may or may not project beyond the end of the body (Figs. 15, 16). The side of the body along which the flagellum runs is thinner than the opposite side.

In a smear of the whole digestive tract, I chanced to obtain some of the "gregarine" type with very thick walls (Plate 5, Fig. 19). Some were spherical and very small, measuring only from 3 to  $4\ \mu$  in diameter (Fig. 20). Others were pear-shaped or elliptical either free or in rosettes (Figs. 19, 21). The trophonucleus, kinetonucleus, and flagellum were plainly visible in the pear-shaped forms, but no flagellum could be found in the spherical individuals. In profile view the flagellum is seen to lie close to the wall. Chromatin granules were often found distributed in the cytoplasm. The wall appears under a magnification of 2,800 diameters to be perfectly homogeneous. Prowazek describes similar forms, but claims that the wall is beaded.

He says that they are cysts which are passed out with the feces and licked up by other flies, thereby causing a new infection.

Out of hundreds of ticks examined there was one exceptional condition found. In this case nearly all the parasites were rounded off, only a few of the typically flagellate forms being present. Occasionally a rounded form was met with among the flagellates of other ticks, but never in predominating numbers (Plate 4, Figs. 10, 11). All stages in the rounding-off process were present. The flagellate bends back on itself at about the middle point of the body (Plate 5, Fig. 28). The concave side thus formed apparently grows shorter and shorter till a rounded body is produced (Fig. 25). In other cases the parasite appears to roll up, the contiguous edges fusing (Figs. 30, 31). The flagellum often passes almost entirely around the body (Fig. 25). In others it curves around over the surface of the parasite and may end with the body wall or project outside as a free whip (Figs. 30, 31). The trophonucleus becomes less distinct while the kintonucleus often nearly equals it in size. Such forms were pictured by Pfeiffer. Other flagellates in the same tick presented still more striking phenomena. In these the anterior end of the flagellum had curled back on itself. A very thin membrane was stretched across the loop to form a disc. The flagellum could be seen as a heavy line circumscribing the disc (Fig. 27). The first stage of this process is shown in Fig. 26. This same condition is said to occur in *Euglena*. In other forms the anterior end assumed the character of a sharp pseudopodium, while in still other cases the end was spread out like the plastic, lobose pseudopodia of some amebae (Fig. 29). These conditions were not the result of faulty technique, for they were observed in the living state and then killed over formol fumes and not smeared on the slide. Associated with these forms was the fact that the wall of the stomach was exceptionally tender. The round forms were marked by lightly staining areas and vacuoles. This fact together with apparent tendency toward nuclear degeneration might be taken as indications of their being involution forms.

Other round forms were found, whose shape later proved to be an artefact. They were present in sections of stomachs killed in Merkel's fluid. Invariably when this killer was used the flagellates crowded very tightly against and between the epithelial cells of the



stomach and rounded off (Plate 4, Fig. 13). The flagellum was sometimes still visible where the parasites were not too close together. The kintonucleus took a peripheral position perpendicular to the wall, which often became slightly thickened. The cytoplasm was clear and resisted staining. The killer which is weak evidently acted so slowly that the flagellates had time to react physiologically and take on those characters which are conducive to their power of resistance. At places where the epithelium was peeling off it was often hard to tell where the parasites left off and the cells began. In many cases I am sure the parasites were within the cells.

Features by which male, female, or indifferent forms could be distinguished were sought for in vain. With the work of Schaudinn (1904) and Prowazek (1904) in mind I spent a great deal of time in looking for forms that could possibly be interpreted as possessing different sex. To be sure in one tick were found large, more or less oval, or gregarine-like forms, which from their size and shape might be considered by the above authors to be females (Plate 4, Fig. 12). They have no more food material than the other forms nor any peculiarities about the nuclei. Some of the small forms may be males, but no evidence for considering them as such could be obtained. In fresh material two cases were found where a small form was clinging to the side of a large one near its posterior end. But at least three interpretations are possible. It may have been an agglutination, a division, or a conjugation of male and female.

The epithelium with the flagellates attached peels off from time to time, but not with the periodicity described by Pfeiffer. He says that the changes in the intestine and the infection of the same were quite similar to those given by Schaudinn for *Culex pipiens*, so that it was unnecessary for him to describe the process. Schaudinn claimed that the cuticular lining of the posterior part of the oesophagus becomes gelatinous and envelops the fresh blood as it passes through the oesophagus into the stomach. After this has taken place the parasites enter upon a period of multiplication. Toward the close of digestion they enter a resting stage and are found attached to the epithelial cells or between them. After the second feeding, and the fresh quantity of blood has become digested they enter upon a multiplication stage and then gradually collect in the anterior part, where



the epithelium is regenerating. They now enter their second resting stage. When the proventricle of the oesophagus is withdrawn from the neck of the stomach the newly formed cuticula with the parasites attached is left behind in the stomach. As the fresh blood is introduced, this mass of parasites attached to the loose cuticle is carried back to the smallest portion of the intestine, where the wall ruptures and the parasites pass into the blood vessels. My observations do not support in any degree Pfeiffer's statement, to the effect that there are changes in the sheep-tick similar to those in *Culex pipiens*.

The tick's digestive tract and habits of feeding are very different from those of the mosquito. There is no proventricle present in the tick, while on the other hand the mosquito lacks a strong valve between the stomach and intestine. In the tick the valve is so efficient that no large masses of material can pass through it. In respect to feeding habits, the mosquito takes a meal and then goes for days or even weeks before getting another, or at least till the blood is digested. Thus there is an alternation between a condition of undigested and of thoroughly digested blood. These conditions do not occur in the tick which spends its whole life on the sheep, both fresh and digested blood always being present in the stomach in the natural state. All ticks have fresh blood in their stomachs when first removed from the sheep. Thus, on a-priori grounds alone, one would be led to the conclusion that these parasites would not behave the same as Schaudinn's. There has been entirely too much generalization by investigators who were anxious to ascribe to all flagellates the stages of life cycle described by Schaudinn. But, sure of so little of the life cycle of the flagellates as we are, one is not warranted in generalizing, merely for a-priori reasons, if for no others.

To be sure, in the sheep-tick portions of the epithelium peel off from time to time and with the attached flagellates pass along posteriorly with the stomach contents. But I can find no alternation between periods of rest and of multiplication. All the forms described, with the two exceptions mentioned, are to be found at the same time in the same tick whether the tick be filled with fresh blood or starved for over a week. Those very long lance-shaped forms (Plate 3, Figs. 2, 3, 4) were found in ticks, one of which was starved 18

hours and the other seven days, yet they cannot be considered as resting forms. There are present the free and the attached forms. The gregarine type peculiar to the intestine is always present in all infected ticks. There are, of course, slight variations in the size and shape of the parasites in various ticks, but these variations are apparently not due to the presence or absence of fresh blood. The epithelium peels off in such a manner that the attached parasites are not rolled up in it. They are thus left so that they can detach and become free in the intestine. The flagellates never pass forward into the anterior third of the stomach whether it is filled with food material or empty. And so in no respect do I find these flagellates behaving like Schaudinn's.

Seasonal changes apparently have no effect upon the flagellates. My observations extended through two years over the months between September and June. In cold weather the ticks remain in the wool on the skin of the sheep, while in hot weather they are found crawling over the surface of the wool. Hence their temperature is probably not effected much by change of seasons.

As regards the method of transmission Pfeiffer made no statement. Presumably he did not examine the blood of the sheep for the parasite. Knowing that insects act as passive carriers, if not as intermediate hosts for trypanosomes, it seemed possible or even probable that these flagellates were sucked up by the tick with the sheep's blood. If such be the case, a very large percentage of sheep must harbor the parasite since practically every full-grown tick is infected. But examinations of the blood, both in the fresh condition and in stained preparations, always showed negative results.

Aware of the hypothesis that *Piroplasma* may have a flagellate stage in an insect host, it occurred to me that I might be dealing in the tick with the flagellate stage of a piroplasma-like form in the sheep, which I had overlooked. Knowing the results of Rogers' experiments on the Kala Azar parasite, I proceeded to make similar cultures of the sheep's blood, with a view to obtain flagellated organisms, provided the tick flagellate had a stage of its life history in the sheep. For a detailed description of these experiments reference can be made to my paper (1908). Suffice it here to say that all sorts of actively motile flagellates and ameboid forms developed, but they

were later shown to be a normal element of the blood, namely, blood-platelets. It was shown conclusively that they have no relation to the tick flagellates, for they were readily obtained in cultures of the blood of the dog, rabbit, and man.

In that paper I threw some doubt upon the truth of the statement of several authors that *Piroplasma* developed into flagellates in sodium citrate culture. It is certain, however, according to Rogers (1907) and Patton (1908) that the parasite of Kala Azar has a flagellate stage. In cultures Rogers obtained rosettes of flagellates exactly comparable to rosettes of *Herpetomonas* and *Crithidia* found in insects. Patton has found further that this parasite develops naturally in the stomach of the bed-bug into a true flagellate, very similar to insect flagellates. On the other hand, there are insect flagellates which probably never enter the blood of a vertebrate, but are transmitted directly from insect to insect. For example the common house-fly, which is not a blood-sucking insect, harbors a *herpetomonas* which, according to Prowazek, is transmitted through the egg and feces. Furthermore, Novy, MacNeal, and Torrey have performed inoculation experiments to ascertain whether flagellates found in mosquitoes have a vertebrate host also. They inoculated the flagellates into sparrows, canaries, pigeons, doves, hawks, owls, crows, rats, and mice, but always with negative results. These experiments, however, as they remark, do not prove conclusively that these parasites do not have a vertebrate host, for there may be some susceptible vertebrate which was not tried. They also found that mammalian trypanosomes soon perish in the stomach of insects, as is shown by the fact that inoculations into fresh animals of the stomach contents of insects a few hours after they have sucked infected blood give negative results. They have further shown that insects do not afford as favorable conditions for the multiplication of mammalian trypanosomes as culture media do. In a letter from Patton I am informed that he failed to trace any development in fleas or lice fed on rats infected with *Tr. lewisi*; also, in fleas and lice fed on a squirrel harboring *Tr. indicum*. He (1907) states further that the *Herpetomonas* which he found in *Culex pipiens* and the *Crithidia* of the water-bug (*Homoptera*) are peculiar to the insects, having "no connection with any blood parasite." He has also made the suggestion to me

that the forms I described (1907) in the rat flea have no connection with the rat trypanosome, but are strictly insect flagellates. Balfour's discovery (1906) of a herpetomonadine flagellate in the flea (*Pulex leopatrae*) and also Patton's discovery of one in the cat flea (*Ctenocephalus felis*) together with my own researches on the tick flagellates lead me to a tentative acceptance of Patton's suggestion.

It has already been stated that the flagellates of the sheep-tick are never found in the fresh blood of the anterior portion of the stomach. This in itself would indicate that the living blood of the sheep is not a favorable habitat for the parasites. Some experiments were made to ascertain whether the flagellates are acquired from the sheep's blood. Pupae of the tick were hatched out and the young ticks thus obtained were fed on rabbit's, sheep's or man's blood. Generally they will not suck for two to four days after hatching. The following table shows the results of these experiments:

TABLE 1.

	Tick	Date of Feeding	Died	Time of Examination	Flagellates Present
Rabbit's Blood	1	Nov. 6	Nov. 9	Nov. 9	None
	2	Nov. 10		28 hrs.	"
	3	Nov. 10		28 hrs.	"
	4	Nov. 10		68 hrs.	"
	5	Nov. 10		24 hrs.	"
Human Blood	6	Oct. 19, Oct. 29	Oct. 31	Oct. 31	"
	7	Oct. 24	Oct. 30	Oct. 30	"
	8	Oct. 28, Nov. 6	Apr. 18	Nov. 9	"
	9	Mar. 28, Apr. 1, Apr. 8, Apr. 13, Apr. 15		Apr. 16	"
	10	Apr. 3, Apr. 5, Apr. 13		" 18	"
	11	Apr. 1, Apr. 11		" 16	"
	12	Apr. 1		" 13	"
	13	Apr. 2		" 13	"
Sheep's Blood	14	Jan. 4, Jan. 5	May 21 Mar. 30	Jan. 7	"
	15	Feb. 1, Feb. 4		Feb. 7	"
	16	May 13, May 17		May 21	Present
	17	Mar. 25		Mar. 30	None

It will be noticed that only one tick developed flagellates. This one had been fed twice on sheep's blood and died within eight days after the first feeding, while some others were fed on human blood, in one instance as many as five times and kept for 19 days, yet possessed no flagellates. At first thought one might think that the data given in this table indicate that the flagellates were obtained from the sheep. But this is not the case. The blood of the rabbit and of man is not a natural food for the tick, and cannot be digested properly by it.

Although the blood is slowly changed and loses its structure, yet it is not digested like sheep's blood as is evidenced by the fact that it becomes hard and caked. Under such conditions it is reasonable to suppose that there is not afforded the nutritive stimulus necessary to bring about a development of the flagellates even though they might be present in the tick. And furthermore, these experiments are not extensive enough to be of conclusive value. Owing to the difficulty in keeping the ticks alive when removed from the host and on account of losing them accidentally much time was consumed in getting the scanty results shown in the table. It is needless to say that even though these experiments had been multiplied indefinitely they would not have given absolute proof on either side of the question. Stronger proof could have been adduced by the use of a fresh-born lamb which had never been bitten by a tick, but such a lamb was not available. If in using such a lamb the ticks should develop flagellates, the evidence would show that they obtained the flagellates from parent ticks but would not prove that the flagellate might not be carried into the blood of a sheep by the bite of an infected tick.

Practically all except the very young ticks were infected. There were, however, a few exceptions. Out of the hundreds of ticks examined, 18 were found uninfected and 16 of these occurred on a single sheep together with seven infected ticks. These facts could hardly be explained if the flagellates are acquired from the sheep. But the reasonable explanation is that these 16 ticks were the progeny of a female which had escaped infection, while the other seven came from infected females. From the negative evidence which has been adduced we are justified in concluding that the sheep does not act as a host for this flagellate.

If this conclusion is correct the method of transmission must be limited to fecal matter or the egg. While infection might be accomplished by a tick taking in some infected fecal matter on the skin of the sheep, it would necessarily be accidental and would rarely take place. The high percentage of infections could hardly be accounted for in this way. Examination of freshly voided feces occasionally showed lively flagellates. But after the feces had dried, although they were placed in salt solution several days, no signs of flagellates, neither of the monadine nor of the gregarine type, were seen. It is conceivable,



however, that forms with a thick wall (Plate 5, Figs. 19-21) might live for a long time even under drying influences and retain their power of infection. With no definite evidence supporting infection through the feces, I believe the only natural mode of infection is transmission through the egg.

The female sheep-tick has two ovaries located laterally in the abdomen, the ducts of which unite to form a common, median oviduct. At the juncture of these ducts there is an enlarged chamber which functions as a sperm receptacle and is generally filled with sperm cells. The other end of the oviduct passes into a dilated portion, which serves as a uterus. The vagina passes from the uterus to the exterior. Milk glands open into the anterior end of the uterus. An egg matures in one of the ovaries, passes out, and after fertilization undergoes partial development in the uterus. The mouth of the developing embryo is near the opening of the milk glands, so that the milk may be sucked in. It might be possible for the young tick to suck up parasites along with the milk, but such a hypothesis was not supported by observation, parasites never having been found in the milk glands. While this embryo is developing in the uterus, an egg in the opposite ovary is maturing and will pass out soon after the expulsion of the embryo. This description of the reproductive system is sufficient for our purposes. The details of the embryology have been worked out by Pratt (1899).

Sections and smears of the ovaries were made with a view of finding the parasites in them. Prowazek was able to demonstrate parasites in the eggs of flies infected with *Herpetomonas*. According to him they are present at first in the copious vitellus where they can be demonstrated only in smear preparations. I have not been able to find the parasites in smear preparations of ova from the sheep-tick. Nor was it possible at first to find them in sections of ovaries killed in Zenker's fluid, which gave the best results for the intestinal forms. After killing with this fluid, the yolk material stains black and obscures the parasites. However, I was always able to find parasites in sections killed with Hermann's fluid and stained with iron hematoxylin. After finding them in these preparations I was able to locate them, though with difficulty, in material killed with Zenker's fluid. Spherical masses, measuring about  $3\mu$ , with a chromatin body lying in a



clear space were commonly found (Plate 5, Fig. 24). It should be noted that these are about the size of the small round forms of the intestine. From these are evidently formed by growth and by divisions of the trophonucleus larger masses with several similar chromatin bodies (Figs. 22, 23). Several of the round forms may be grouped together in irregular masses. They are always very distinct from the cytoplasm and have a very definite structure. They bear a strong resemblance to some of the forms found by Prowazek in the vitellus of the fly's egg (see Prowazek, 1905, Fig. 6, *d*). In these, he says, the nutritive nucleus perishes, while the kintonucleus undergoes numerous divisions. He believes, therefore, that these forms perish, as he was not able to trace any further development of them. He figures the kintonuclei as round or rod-shaped bodies lying in clear spaces. In many of my forms the chromatin bodies, which are very definite, vary from minute round granules to large, oval, or round masses. In one form (Fig. 22) there were indistinct, rodlike bodies at the edge and on the surface of the mass which might be interpreted as kintonuclei. I am more inclined to believe that they were artefacts, little wrinkles in the surface, for they were not found in other forms. I cannot say whether the other chromatin bodies are trophonuclei or kintonuclei. Possibly the larger ones are trophonuclei, the smaller ones kintonuclei. When the forms represented by Figs. 22 and 23 were greatly destained the large chromatin bodies appeared as mere granules like the central granule in the trophonuclei of intestinal forms. And so, contrary to Prowazek, I am of the opinion that the large masses are trophonuclei. There can, however, be little doubt that these foreign masses are parasites which infect the progeny. The egg requires about a month for its development, and hence it is exposed for a long period to the possibility of infection. This perhaps explains why a tick is seldom found without infection. The further development of these forms I have not followed. Neither by sectioning nor by teasing the viscera in salt solution, was I able to find the parasites in freshly hatched ticks.

There are various opinions as to what characters belong to the genera *Crithidia* and *Herpetomonas* respectively. Léger (1902) described a flagellate occurring in the region of the malpighian tubes in the gut of *Anopheles maculipennis* for which he formed a new genus,

*Crithidia*. These forms are small, measuring from 3 to 10  $\mu$  in length. The kintonucleus is anterior to the trophonucleus and in the longer forms a rudimentary undulating membrane is present. The shorter forms are truncated and have no undulating membrane. In 1904 he described a flagellate occurring in the Tabanidae under the name *Herpetomonas subulata*. In this case he recognized both the long, slender and the short, pear-shaped, or truncated type. Here again the long forms, several times longer than *Crithidia*, have an undulating membrane, formed by the body being drawn out along the flagellum. The membrane is more marked than in his *Crithidia*. The short forms are found attached in layers to the wall of the intestine in the region of the malpighian tubes.

Patton (1907) described a flagellate in the water-bug under the name *Crithidia*. The monadine form resembles very closely the monadine forms of Léger's *Herpetomonas subulata*. In the same paper he figures under the name *Herpetomonas* a flagellate in *Culex pipiens*, the monadine form of which has a close resemblance to the monadine form of *Herpetomonas jaculum*, described by Léger (1902). These forms have no undulating membrane, and the kintonucleus is near the anterior end. It will be seen, therefore, in comparing these forms, as has already been pointed out by Léger himself and by Novy, together with his associate workers, that the distinctions between the two genera are at best fragile. The forms described in this paper only add to the fragility of distinction.

Prowazek (1904) places in the genus *Herpetomonas* two species of flagellates; one from the gut of the common house-fly *Musca domestica*, the other from the meat-fly *Sarcophaga haemorrhoidalis*. In these forms he finds two terminal flagella with a delicate membrane stretched between them. They take origin in the kintonucleus which is located in the anterior end of the parasite, and from which a double, twisted thread passes to the posterior end where it ends in a double granule, *diplosome*.

Lühe (1906) holds that the difference in size between the short crithidian type and the long herpetomonadine form does not justify the formation of two genera. He would, however, retain both genera and characterize *Crithidia* as possessing a kintonucleus near the trophonucleus, a single flagellum, and a slightly developed undulat-

ing membrane, while *Herpetomonas* would be characterized by a terminal kinetonucleus and a double flagellum.

Novy, MacNeal, and Torrey believe that Léger in the case of *Herpetomonas subulata* was dealing with both genera, the small, pear-shaped, truncated forms belonging to the genus *Crithidia*, and the long forms to *Herpetomonas*. Furthermore, the *Crithidia minuta* which Léger later placed in the genus *Herpetomonas* because of the presence of a long form, they believe to be a true *Crithidia*. Their reason for this belief is that in cultures *Crithidia* and *Herpetomonas* have distinct characters and always retain them, never changing from one into the other. They further refuse to accept Lühe's classification until it has been confirmed that the *Herpetomonas* described by Prowazek always possesses a double flagellum. While they regard these two genera as representing primitive trypanosomes and hence to be placed in the genus *Trypanosoma*, yet, as long as the genera are retained, they would characterize them in the following way:

"The genus *Crithidia*, if it is to be retained, is characterized by peculiar, short oval or pyriform bodies which are usually round or obtuse posteriorly, while the anterior end is truncated, or even slightly depressed, and bears a short, straight flagellum. In this short form the nucleus is near the posterior end, and the blepharoplast is by its side. Somewhat longer cylindrical forms, tapering very slightly, or rounded at each end, may be present, and these are provided with a long flagellum. In this form the nucleus is near the center, and the centrosome is usually about midway between the nucleus and the anterior end. Both forms may occur in rosettes with flagella directed centrally. The undulating membrane and posterior diplosome, as seen in the *Herpetomonas*, are absent."

This characterization is based upon the results obtained from cultures, in which crithidian and herpetomonadine forms, as characterized above, retained respectively those characters throughout numerous sub-cultures. These authors claim to have found that mosquitoes may be infected either with *Crithidia* or *Herpetomonas*, or with both genera at the same time.

It is clear that the flagellates of the sheep-tick could be put under neither genus according to these authors' characterization. Their *Herpetomonas* has an undulating membrane and a diplosome. The longer forms from the sheep-tick have a distinct undulating membrane but no diplosome. *Herpetomonas*, they say, gradually tapers posteriorly. These forms, as I have stated before, are typically blunt at the posterior end. The short pear-shaped forms would go all right

under *Crithidia*, but the longer monadine forms would not, since they possess an undulating membrane. If I am dealing with two genera, as they believe concerning Léger, then the short pear-shaped forms occurring in the intestine could be placed under the genus *Crithidia*, and for the longer type occurring in the stomach anterior to the malpighian tubes, a new genus would have to be created. But, according to Lühe, all these forms could be put in the genus *Crithidia*. It is very improbable that there are two different genera present. All gradations from the very long flagellate with a conspicuous membrane down to the small form with obscure membrane are to be found. Going from the anterior toward the posterior part of the stomach one can see in a general way a gradual reduction in the length and size of the forms. Passing through the valve into the intestine one comes to the true crithidian type. Here and there among the truncated forms attached to the wall of the intestine will be found forms that are intermediate between the truncated type and the more typically herpetomonadine forms found in the stomach. Rosettes in the intestine, as they are forming, are seen to be made up of individuals representing all stages from the purely herpetomonadine type down to the small crithidian form. While the latter type is never found in the stomach, yet the connecting forms are sufficiently numerous to establish their generic identity. And further, if the forms in the intestine belong to a different species from those in the stomach, it would be hard to account for the fact that both species are always associated. According to the laws of chance one would expect that occasionally only one of the species would be found. On the contrary, either both are present or both absent. Considering them as belonging to the same species these facts could, however, be accounted for on physiological grounds. Owing to the different degrees to which digestion and absorption have proceeded at successive levels along the digestive tract, one would expect to find certain differences in the morphology of the parasites correlated with these physiological differences. At the valve separating the stomach and intestine there is necessarily a much more marked physiological change than anywhere else. The end of the stomach narrows down to a very small caliber while the end of the intestine just on the other side of the valve is comparatively large and funnel-shaped. These marked anatomical changes must produce just as

strong physiological differences, which are capable of modifying the flagellates as they pass from the stomach into the intestine. The crithidian type in the intestine is not found anterior to the valve because it would be mechanically impossible for them to pass anteriorly through it. The herpetomonadine type is found in the intestine because of the natural passage of stomach contents into the intestine. The parasites that are not passed out directly with the feces are doubtless modified into the crithidian type. Hence I conclude that only one species is present and that it belongs to the genus *Crithidia*, provided this genus is to be retained as characterized by Lühse. Mention has already been made of the fact that Patton has placed a very similar flagellate in the same genus.

As a matter of fact the distinctions between these genera are not sufficiently strong to receive generic rank but should rather be considered as specific. This form from the sheep-tick is intermediate between the two genera. In the possession of a very marked undulating membrane, this form unquestionably stands in close relation to the trypanosomes. By means of the cultural method Novy and his associates have pointed out the close affinities between insect flagellates and trypanosomes, contending that the former are primitive trypanosomes and that in cultures the trypanosomes revert in structure to the more primitive form. They would go so far as to place them all in the genus *Trypanosoma*. Granting, as I do, that they are correct in their contention that insect flagellates represent the primitive type of trypanosomes, it still seems to me that the modifications of the trypanosomes in structure and behavior, due to the marked change in habitat on account of being introduced into the blood of vertebrates, are strong enough differences to receive generic rank. The grounds for final retention or rejection of the genera *Crithidia* and *Herpetomonas* must be sought in their life histories. The genera cannot be intelligently characterized until the life histories have been followed out. If but one genus, *Trypanosoma*, is to be retained, a much closer agreement in the life cycles of insect flagellates and trypanosomes must be found, than is indicated by the knowledge we already have of their life histories. According to our present knowledge there are certainly much greater differences between trypanosomes and insect flagellates, than exist among the various species of insect flagellates or of trypanosomes.



If we accept the view that insect flagellates are the ancestors of the trypanosomes, it is easy to explain the origin of the undulating membrane. In respect to the trypanosomes the belief has been held that the membrane was formed by the fusion with the body of a second flagellum which was left to drag behind. Such an assumption is entirely unwarranted and is not supported by any morphological evidence in the trypanosomes. There is no indication of there being at present or of there ever having been a second flagellum. On the other hand, if we take a flagellate without a membrane, like *Herpetomonas jaculum* represented by Léger (1902, Fig. 12), as the primitive type, we can find all stages of development between this and the trypanosomes. In this form the kinetonucleus is near the end of the body and hence there is no reason for the existence of a membrane. Owing to the vibrations of the flagellum which must be transmitted more or less to the anterior portion of the body, there would be a tendency for the end of the body to become more flexible and membranous. Suppose the kinetonucleus be carried farther back as in the crithidian type, this effect would be intensified so that quite a definite membrane bordered by the flagellum would be produced. Now suppose the kinetonucleus be carried still farther back even posterior to the trophonucleus as in the trypanosomes, then it is clear that a strongly developed membrane would be formed along the body as far back as the point at which the flagellum passes into the body. Ontogenetically the position of the kinetonucleus is variable. In cultures of the trypanosomes it is found to pass from the posterior region to the anterior. Moreover, the membrane and flagellum are unstable, being entirely lost at times. Since such ontogenetic changes as these occur, it is not presumptuous to suppose that such phylogenetic changes as outlined above may have taken place. The changes are, therefore, to be regarded as progressive from a form having a single flagellum attached to the anterior end up to the trypanosomes which possess a well-developed undulating membrane bordered by a single flagellum. With their introduction into the blood, the mechanical consistency of their new habitat necessitated the development of a strong locomotor apparatus such as is now found in the blood trypanosomes.

My sincere thanks are due to Dr. Henry B. Ward for his direction



of this investigation which was carried on in the zoölogical laboratory of the University of Nebraska. His continued suggestions and criticisms have been very helpful in the work. I am also indebted to Dr. Joseph H. Powers for valuable suggestions.

Since the writing of this paper an extensive article by Minchin (1908) has appeared. He discusses the development of *Trypanosoma gambiense* and *Tr. grayi* in *Glossina palpalis*. Among *Tr. grayi* he found herpetomonas-like forms, which were without undulating membrane and had a kinetonucleus anterior to the trophonucleus. A comparison of his figures (Plate 11, Figs. 162, 164, 169, and 170) with mine (Plate 3, Figs. 1, 2, 3, and 4) will be sufficient to convince one of the marked similarity in structure. The formation of cysts in the proctodaeum as he describes and figures it (Plate 12, Figs. 186-200) is precisely similar to the process in the sheep-tick. He at first considered *Tr. gambiense* and *Tr. grayi* as identical but later came to regard them as distinct species. When this point was reached, he naturally agreed with Novy that *Tr. grayi* is a parasite of the fly. But after further reflection he has come to regard it as a parasite having a vertebrate host, probably, avian. I believe, nevertheless, that these herpetomonas-like forms, which are so similar to my *Crithidia* that is peculiar to the insect, *Melophagus ovinus*, may be insect flagellates and have no connection with the trypanosomes.

I have never been able to see a granule (blepharoplast, according to Minchin) at the base of the flagellum and separate from the kinetonucleus. In some cases the flagellum stains right up to the kinetonucleus, in others a portion remains unstained.

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#### EXPLANATION OF PLATES 3, 4, and 5.

FIG. 1.—Large monadine type from stomach of tick 40 hours after removal from sheep.  $\times 2800$ .

FIG. 2.—A typical monadine form from the stomach of tick 68 hours after removal from sheep. Seven chromatin granules and a central granule are apparent.  $\times 2800$ .

FIG. 3.—Monadine form from stomach of tick starved seven days.  $\times 2800$ .

FIG. 4.—Lance-shaped monadine type from stomach of tick starved seven days. Posterior end sharply pointed.  $\times 2800$ .

FIG. 5.—Short, thick, monadine form from stomach of tick 40 hours after removal from sheep.  $\times 2800$ .

FIG. 6.—Short monadine type from stomach of tick 52 hours after removal from sheep. The trophonucleus possesses 11 chromatin granules.  $\times 2800$ .

FIG. 7.—Monadine type from stomach of tick 72 hours after removal from sheep. Trophonucleus contains 11 chromatin masses, kinetonucleus dumb-bell shaped.  $\times 2800$ .

FIG. 8.—Monadine type with posterior end contracted into a ball. From stomach of tick starved seven days. Four chromatin masses and central granule visible.  $\times 2800$ .

FIG. 9.—Flagellate in process of division. From stomach of tick 26 hours after removal from sheep.  $\times 2755$ .

FIG. 10.—A round form with flagellum still present, from stomach of tick 52 hours after removal from sheep.  $\times 2755$ .

FIG. 11.—Round form from the stomach of a tick starved three days.  $\times 2755$ .

FIG. 12.—Female (?) from stomach of same tick as Fig. 10.  $\times 2755$ .

FIG. 13.—Portion of cross-section of stomach of tick 40 hours after removal from sheep. Round parasites clinging to the epithelial lining. The form due to the effect of Merkel's killing fluid.  $\times 650$ .

PLATE 3.





PLATE 4.



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FIG. 14.—Section through intestine cutting the posterior part of valve. Forms transitional between the long herpetomonadine type of the stomach and the short crithidian type of the intestine.  $\times 1445$ .

FIG. 15.—Truncated form from the intestine of tick 12 hours after removal from sheep.  $\times 2755$ .

FIG. 16.—Pear-shaped form with flagellum projecting beyond the body. From intestine of tick 18 hours after removal from sheep.  $\times 2755$ .

FIG. 17.—Rosette of pear-shaped forms from the rectum of tick 18 hours after removal from sheep.  $\times 2755$ .

FIG. 18.—From same section as Fig. 14. Parasites on the sides of the intestine. In this region the intestine is always dilated, its caliber being several times that of the posterior end of the stomach. The kinetonuclei of the basal layer are anterior to the trophonuclei, those of the outermost layer posterior to the trophonuclei, while those of the middle layers are intermediate between the two extremes.  $\times 1445$ .

FIG. 19.—Pear-shaped cyst from tick 40 hours after removal from sheep, probably from intestine or rectum.  $\times 2825$ .

FIG. 20.—Round cyst from same tick as Fig. 19. Flagellum absent.  $\times 2825$ .

FIG. 21.—Rosette of cysts from same tick as Figs. 19 and 20.  $\times 1180$ .

FIG. 22.—Parasite from the vitellus of the egg of the tick. Throughout the cytoplasm are clear areas containing chromatin bodies (probably trophonuclei). At the edge are rodlike structures (possibly kinetonuclei).  $\times 3030$ .

FIG. 23.—Parasite similar to Fig. 22, but lacking the rodlike structures. From the same egg.  $\times 3030$ .

FIG. 24.—Simple round parasite from another egg. In this one the nucleus was not so homogeneous in structure. An indefinite rodlike structure (kinetonucleus?) just above and partially to one side of the trophonucleus.  $\times 2825$ .

FIGS. 25-31.—Peculiar involution (?) forms from the stomach of tick 40 hours after removal from sheep. Figs. 25 and 28 show early stages in the rounding-off process. Figs. 30 and 31 show the final result. Figs. 26, 27, and 29 show the plastic condition which the flagellum and anterior end of the body assume. In Fig. 26 the anterior end has bent back on itself and a membrane is stretched across the loop thus formed. In Fig. 27 this condition is carried still farther. In Fig. 29 the flagellum appears on one side as a definite line.  $\times 2825$ .



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## THE FECAL BACTERIA OF HEALTHY MEN.\* PART I. INTRODUCTION AND DIRECT QUANTITATIVE OBSERVATIONS.†

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### CONTENTS.

INTRODUCTION.—Conditions of the investigation. Purpose of this paper. Divisions of the subject.

QUANTITATIVE METHODS.—The preparation of a suspension of the fecal bacteria. The microscopic counting procedures. The gravimetric determination. The calculation and application of these determinations. The differential count of mixed fecal flora.

QUANTITATIVE RESULTS.—The number of bacteria in feces. The weight of bacterial dry substance in feces. The relation of these to each other. The daily excretion of bacteria. Fecal nitrogen contained in the bacteria. The variety of fecal bacteria recognizable by microscopic examination.

SUMMARY.

REFERENCES.

### INTRODUCTION.

THE fundamental importance of bacteria in the physiology of the digestive tract of vertebrates is quite generally recognized. The researches of Nuttall and Thierfelder,<sup>18</sup> Schottelius,<sup>21</sup> and Mme.

\* Received for publication February 10, 1909.

† The culture experiments will be reported in Part II, which will appear in a short time.

Metchinkoff<sup>17</sup> still leave unsettled the question, suggested by Pasteur,<sup>19</sup> whether animal life could continue without their presence. For the pathologist, intestinal bacteriology has long been a fertile field and the researches relating to it are very numerous indeed. We shall not attempt a citation or any review of the voluminous literature of this subject as these may be found in the monograph on the micro-organisms of the feces by Strasburger.<sup>20</sup> Only the researches of others which are closely related to our own work will be considered briefly in the description of our methods and results.

During the past year this laboratory undertook an experiment to determine the influence of cured meats upon human health. In connection with this experiment it was deemed advisable to study the fecal flora, inasmuch as this study might prove of value as an index to the condition of the digestive tract and of possible alterations due to different diets. It was also recognized that here was afforded a somewhat exceptional opportunity for the study of the micro-organisms of the feces of normal adult men during a long period of time. No very comprehensive examination of the fecal flora in a group of normal adults seems to have been previously undertaken.

It is our purpose to present, in this communication, the methods of examination employed, together with the results obtained, wherever these appear to be of value as a contribution to the physiological or pathological bacteriology of the intestine. During the course of the work 266 stools were examined. About three-fourths of these serve as the basis of this paper.

#### SOURCE OF MATERIAL.

The observations were made upon 12 men, university students from 19 to 30 years of age, living together in a club-house specially provided for the metabolism experiment. They were fed exclusively at the club table upon weighed quantities of foods, the composition of which was determined by chemical analysis. The menu provided was similar to that of a student boarding-house or club of the better grade, with considerable variety of diet from day to day. The breakfast consisted of fruit, cereal, meat, potatoes, bread, butter, water, and milk or coffee. For luncheon, a soup, cereal, fruit, bread, butter, water, and milk or tea were served. Dinner was served in the evening. It consisted of meat, potatoes, a second vegetable, a pudding, bread, butter, water, and milk or cocoa. For the sake of variety, various fruits, cereals, soups, vegetables, and meats were used, and the potatoes were prepared in various ways for the different meals. These variations were all made in accordance with a definite schedule of meals. This same schedule was exactly repeated every eight days. A certain amount of selection was allowed to each individual subject, as to the quantity of the different foods he



should consume, as long as this choice did not interfere with the proper relation between food intake and excretion. For example, Subjects K and L were the only ones who chose to take coffee; Subjects H and I did not eat the second vegetable dish at dinner after March 16; Subject E ate only one-half the usual amount of meats after January 12, and Subject F, only three-fourths the usual amount of meats during the whole period of observation; Subject B ate none of the cereals. These variations are such as would ordinarily occur on account of individual tastes. There was also a variation in the quality of the meats consumed by the different subjects during a part of the period of observation. Some of the subjects during this time received cured meats as part of the meat ration, while others received only perfectly fresh meats during the entire period of observation. The quantity of cured meats and of meat preservative consumed was in no case greater than that which may ordinarily be consumed in boarding-houses or clubs. At least half of the meats served to each individual were fresh and free from preservatives. Thus the diet with its variations represents fairly well an ordinary mixed diet.

A detailed discussion of the diet would lead beyond the scope of the present paper. It is not our purpose to consider the relation of the fecal flora to variations in the diet, this question being reserved for another time. For the present, the variations in diet may be regarded as normal and the results of the bacteriological examinations regarded as fairly representative of normal intestinal conditions.

#### PLAN OF THE WORK.

Our observations fall in general under two heads: first, the direct examination, including the quantitative determination of the bacteria in the feces by microscopic enumeration and by the gravimetric method of Strasburger,<sup>23</sup> and the determination of the relative numbers of morphologically different bacteria present by microscopic study of Gram stained films; second, the culture experiments, which were carried out in a quantitative manner in each case, (*a*) to determine the number of bacteria capable of development on various media in the air and in a hydrogen atmosphere, and to identify these species as far as possible; (*b*) to determine the fermentative activity of the mixed fecal flora upon various sugars, as suggested by Herter;<sup>8</sup> (*c*) to bring to development by special methods the different species of bacteria present, which fail to develop upon ordinary plates.

#### COLLECTION OF MATERIAL.

Each stool was passed directly into a sterilized agateware basin, provided with a cover, no special precautions being taken to avoid contamination during passage through the anal canal, but all contamination of the material after passage being carefully excluded. As a rule the material was delivered at the laboratory within ten minutes after passage and the examination of it begun at once. In exceptional cases immediate examination was impossible and then the material was kept cold by standing the pan on a block of ice in the ice box until examined.

#### PREPARATION.

The weighing, macroscopic examination, and thorough mixing of the feces were performed as rapidly as possible. Ordinarily a fairly good mixture was obtained by rubbing the feces with a sterile porcelain spatula in the collecting basin. At times the material was transferred to a large mortar and mixed with a pestle. The difficulty experienced in getting a satisfactory mixture varied considerably with different stools,

and in no case could the final mixture be regarded as very uniform, as macroscopic bits of vegetable tissue 1 to 5 mm. in length commonly remained undivided. However, the error due to this factor was not very serious because the sample taken for examination was fairly large.

#### BACTERIOLOGICAL SUSPENSION.

Immediately after the mixing was completed a 1:100 suspension of the feces was prepared to serve as the basis for the general bacteriological examination. This required considerable care in its preparation but the method we have used is very simple and the resulting suspension uniformly satisfactory. In order to get a uniform suspension of the fecal bacteria it is important that sufficient diluent be used. The suspension of 5 gm. of feces in 50 c.c. of liquid we found to be very difficult but the suspension of one-half gm. feces in 50 c.c. was found to be a relatively simple matter. The procedure is as follows: A perfectly clean, dry, sterilized, glass-stoppered measuring flask of 50 c.c. capacity is first accurately weighed on the analytical balance. Then by means of a sterile glass rod about 4 mm. in diameter and somewhat pointed at the tip, 500 mg. mixed feces is transferred to the flask and deposited on the inside of the neck, the quantity being subsequently increased or diminished as necessary until the required amount is obtained. The weight is observed with the glass stopper in position and an amount of feces within 1 mg. of the desired weight is considered sufficiently accurate. Rapid manipulation is essential to avoid error from loss by evaporation. About 10 c.c. of sterile 0.75 per cent salt solution is now transferred to the flask by means of a Pasteur bulb pipette and the feces rubbed up in the neck of the flask with another clean sterile glass rod. Further salt solution is then added, the glass rod and the neck of the flask being washed with it as it runs in, until the flask contains about 20 c.c. The flask is then stoppered and vigorously shaken for a minute, then inverted so that unsuspended solid particles settle into the neck against the glass stopper. The flask is then slowly erected so that the liquid is decanted from the neck back into the body of the flask, leaving the solid bits in the neck where they are now easily crushed with the glass rod. The flask is now again shaken and the solid particles again allowed to settle into the neck, this procedure being repeated until there remain no macroscopic bacterial masses, recognized as opaque brown lumps. The visible pieces of tissue will then be washed clean so that their character is easily recognized and the bacteria have been washed away and suspended in the liquid. The flask is now violently shaken for 3-5 minutes to break up microscopic bacterial clumps. Further salt solution is then added up to the 50 c.c. mark, the glass rod and the neck of the flask being washed clean during the process, and after a final vigorous shaking the suspension is ready for use. It must always be thoroughly remixed immediately before measuring out portions from it.

The preparation of such a suspension requires 10 to 20 minutes of careful work but after a little practice the result is uniformly satisfactory, and microscopic examination shows the individual bacteria well separated. This suspension is used for the enumeration of the total bacteria, for the differential counting of the different types, and for all the culture experiments. One c.c. of it represents the bacterial content of 10 mg. feces.

#### QUANTITATIVE DETERMINATIONS

As is well known the plate method is of no value for the estimation of the total quantity of bacteria present in feces, as the number

of bacteria here capable of development on plates is only a small fraction of the total quantity present, and bears no constant relation to the latter number. The total quantity of bacteria was determined by three separate methods, the microscopic counting method of Winterberg,<sup>25</sup> the Eberle<sup>5</sup>-Klein<sup>10</sup> counting method, and the gravimetric method of Strasburger.<sup>23</sup>

#### THE EBERLE-KLEIN METHOD.

Eberle,<sup>5</sup> working in Escherich's laboratory, was the first to determine the quantity of fecal bacteria by counting them in stained films. His experiments were carried out with normal infants' stools. Coverglass preparations were made from fecal suspensions of known dilution and after being completely dried were stained with freshly prepared aniline water fuchsin, then washed in water, air-dried, mounted in Canada balsam, and counted.\* The method was very much improved by Alex. Klein<sup>10</sup> and Hehewerth.<sup>6</sup> Klein had noticed that vegetative bacteria were more sensitive to disinfectants in a moist, than in a dry state, and this led him to believe that bacteria would also be stained more readily in a moist condition than after drying. Therefore he allowed the dye to act while the bacteria were suspended in a liquid and the coverglass preparation was made, dried, and fixed, only after the bacteria were stained. Later the same investigator<sup>12</sup> modified the method still further, by using gelatin to fix the stained bacteria to the coverglass. The aqueous solution of gelatin and the stained bacterial emulsion were put upon the coverglass separately, then mixed and spread. When dry the preparation was immediately mounted in Canada balsam without flaming.

The procedure as we have employed it has been considerably modified. In the addition of the gelatin to the bacterial suspension, in spreading the films, in selecting the fields to be counted, and in counting individuals, rather than groups, as units, the technic differs from Klein's method. Of the 1:100 suspension of the feces prepared as described above (p. 126), 2½ c.c. is transferred to a clean dry bottle, ½ c.c. of melted nutrient gelatin and 2 c.c. of aniline water gentian violet added, and the whole thoroughly mixed and allowed to stand for 3-5 minutes. Then by means of a platinum loop, the carrying capacity of which has been previously determined with great care, a loopful of the mixture, well shaken immediately before, is transferred to a clean, flamed 20 mm.

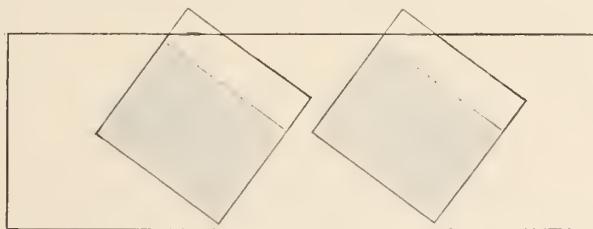


FIG. 1.—Coverglasses mounted so that the proper diagonals of the bacterial films lie in the longitudinal diameter of the slide.

square No. 1 coverglass and deposited near the center of the glass. Immediately another coverglass of the same size is accurately placed on top of the first so that the two glasses

\* Winslow<sup>24</sup> has employed a similar method in estimating the number of bacteria in sewage.

are in contact over about  $\frac{3}{4}$  of their surfaces and the sides evenly fitted together. As soon as the liquid has spread evenly between the two coverglasses, they are quickly slipped apart and allowed to dry. The technic here is the same as ordinarily used in the preparation of coverglass blood films. If the preparations do not appear evenly spread the process is to be repeated with two more coverglasses until a satisfactory result is obtained. The films are next accurately measured by a millimeter rule and then, without further treatment, mounted in Canada balsam upon one slide so that one diagonal of each rectangular film is parallel with the long axis of the slide. By laying the slide over co-ordinate paper these diagonals are readily brought into the same line parallel with the edge of the slide, as shown in Fig. 1.

The covers must be so mounted that these diagonals now in a straight line are those which crossed each other as the two coverglasses were originally placed together in preparing the films. When properly made each diagonal measures almost exactly 25 mm.

For counting the bacteria in the preparation, the Leitz  $\frac{1}{2}$  oil immersion objective and the No. 3 ocular fitted with an Ehrlich ocular square to restrict the field to a convenient size, and a mechanical stage graduated in millimeters are employed. Beginning at the end of the diagonal of one coverglass the bacteria are counted in each of 25 fields 1 mm. apart along this diagonal. In a similar way 25 fields are counted on the diagonal of the second coverglass, making a total of 50 fields, the average of which may be considered as representative of both films. The size of the square field is accurately measured by a stage micrometer. From the data then at hand the number of bacteria per milligram feces is calculated.

*Example.*—B 252, Subject H, July 15, 1908. In the preparation made as described each film measures  $16.5 \times 17.5$  mm., total film area therefore,  $33 \times 17.5$  mm.; the field employed measured 0.0445 mm. square; the amount carried by the loop 2.01 mg.; the number of bacteria counted in 50 fields was 559 and the original 1:100 suspension was used (diluted to 1:200 by dye and gelatin). From these data,

$$\text{Bact. per mg. feces} = \frac{559 \times 33 \times 17.5 \times 200}{50 \times 0.0445 \times 0.0445 \times 2.01} = 324,000,000.$$

In this fraction all the members except the size of the films and the number of bacteria counted may be kept constant and the calculation simplified by use of logarithmic tables.

With apparatus and reagents ready and the 1:100 suspension prepared, this entire estimation can be completed in about 40 minutes. The results cannot be considered very accurate as the platinum loop does not carry an exactly constant quantity. There is also sometimes great difficulty in distinguishing micrococci from other fine particles in the preparations. The concentration of the bacterial suspension also influences the final result. The estimation is in general relatively higher when dilute suspensions are counted as we have observed in applying it to enumeration of bacteria in pure cultures.

#### THE WINTERBERG METHOD.

The first employment of the Thoma-Zeiss blood-counting chamber for bacteriological technic was made in the one-cell dilution method of obtaining a pure culture. By this method the number of cells per unit volume of a suspension could be ascertained and the dilution required to obtain a suspension with one cell in two to five drops calculated.<sup>9</sup> In this way the first pure bacterial culture was obtained.<sup>13</sup> Heinrich Winterberg<sup>25</sup> was the first to use this method of bacterial counting, and to test its accuracy both as regards suspensions of varying dilutions and in comparison with the



microscopic-plate counting method. He considers the method more accurate than the plate counting method, but as a quantitative procedure he would consider it unimportant. He says his determinations were too low. Winterberg's counts were made with suspensions of living bacteria in bacteria-free distilled water.

The method, as we have used it, is as follows: a portion of the 1:100 suspension of feces is diluted 10 times and a portion of this is drawn up to the mark 1 in the capillary of a diluting pipette, ordinarily used in estimating the white blood cells. This is diluted to the mark 11 with a dilute solution of methylene blue in physiological-salt solution. (The staining solution consists of methylene blue 1 gram, glycerin 25 c.c., distilled water 75 c.c. A few drops of this are mixed with 10 c.c. of 0.8 per cent salt solution until the mixture is well colored but not too opaque. This mixture is used as the diluting fluid. A little practice will show the proper depth of color to be employed.) The suspension is thoroughly mixed in the bulb by shaking and rolling in the usual manner. Several drops are blown out and then a very small drop is placed in the center of the circular elevated portion of the slide, which has been previously thoroughly cleaned by washing in distilled water and alcohol. A clean, thin, ground cover-slip is made slightly moist by breathing upon it and is immediately placed upon the slide. Slight pressure upon it causes the Newton color rings to appear and these remain after the pressure is removed if the preparation has been properly made. The slide is allowed to stand one to two hours to allow the bacteria to settle. Then the bacteria in 50 small squares upon the marked scale are counted microscopically, the No. 7 Leitz objective and No. 3 ocular being used. The calculation is simple as an example will show.

*Example.*—B 252, Subject H, July 15, 1908. 50 squares contain 400 bacteria. Therefore the average per square is 8.0 bacteria. One small square =  $\frac{1}{4000}$  c.mm. One c.mm. contains  $8 \times 4000 = 32,000$  bacteria. One c.c. contains  $32,000 \times 1,000 = 32,000,000$  bacteria. Pipette dilution = 1:10.  $32,000,000 \times 10 = 320,000,000$  bacteria per c.c. of  $\frac{1}{1000}$  suspension. One c.c. of  $\frac{1}{1000}$  suspension is equivalent to 1 mg. feces. Therefore there are 320,000,000 bacteria per mg. feces.

Counting by this method is an exacting process and much practice is required to see all the bacteria present. Careful adjustment of the light is important and best results have been obtained by illumination with the Welsbach light; constant focusing through the different layers is necessary. Perhaps the greatest source of error is the difficulty of distinguishing accurately the bacteria. Skill in this is acquired only by considerable practice. The method has the advantage of simplicity. Our results seem to confirm those of Winterberg in that high dilutions give a relatively high count.

#### GRAVIMETRIC DETERMINATION.

The quantitative determination of the bacteria by the gravimetric method is essentially the procedure of Strasburger.<sup>23</sup> Immediately after making up the 1:100 suspension this determination is begun. To determine the dry substance we weigh out quickly two portions (2 to 3 gm.) of feces in weighed porcelain crucibles and dry to constant weight at 102° to 103° C. Two similar amounts (2 to 3 gm.) are also weighed out in weighed centrifuge tubes. The weighings are done as rapidly as possible and read accurately within two milligrams. Our method here differs from that of Strasburger, who employs a burette to measure off exactly 2 c.c. for each determination, and from that of Steele<sup>22</sup>, who uses a pipette to measure off 5 c.c. of a homogeneous suspension of the whole stool diluted with salt solution. We also consider it quite desirable to do

the determinations, both of the dry substance and of the bacteria, in duplicate. Weighing the material in the centrifuge tubes saves time and avoids loss in transferring. Each tube is carried through as a separate determination.

The bacteria are separated from the remaining fecal substance by a process of fractional sedimentation in the centrifuge. To the feces in the centrifuge tube a few drops of  $\frac{1}{2}$  per cent hydrochloric acid are added and mixed to a smooth paste by means of a glass rod. Further amounts of the acid are added with continued stirring until the material is thoroughly suspended and the tube nearly full. The tube is then whirled in the centrifuge at high speed for  $\frac{1}{2}$  to 1 minute. We have found the electric centrifuge to be the most satisfactory. By this treatment the suspension is sedimented into more or less definite layers, the uppermost of which is fairly free from the larger particles while it still contains a considerable quantity of the bacteria. The upper three-fourths of the suspension are now drawn off by means of a Pasteur bulb pipette and transferred to a beaker properly labeled. The sediment remaining in the tube is again rubbed up with the glass rod, which is kept in the beaker during the centrifugation, with the addition of further dilute acid, and again centrifugated for  $\frac{1}{2}$  to 1 minute. The supernatant liquid is pipetted off, and added to the first, the same pipette being used for the one determination throughout. A third portion of the dilute acid is then added to the sediment, again mixed by stirring and by vigorous shaking, and again centrifugated. In this way a third, fourth, and eventually a fifth washing is obtained, all being added to the first. During the process care is taken to wash the material from the walls and mouth of the centrifuge tube down into it. Finally when the sediment is sufficiently free from bacteria, the various remaining particles are visibly clean,\* and the supernatant liquid after centrifugation remains almost clear. The pipette is thoroughly rinsed out with this last portion which is then added to the preceding fractions in the beaker. We now have in the beaker practically all the bacteria present in the original portion of feces and in the centrifuge tube a considerable amount of the other solid matter. The latter is discarded. The suspension in the beaker is now transferred to clean centrifuge tubes and centrifugated at high speed for a minute and the supernatant liquid transferred to a clean beaker by means of the pipette. The tubes are then refilled from the first beaker and thus all the suspension centrifugated a second time. The first beaker is finally carefully washed with the aid of a rubber-tipped glass rod, the second sediment in the centrifuge tubes washed free of bacteria by means of this wash water and by successive portions of dilute acid, and the supernatant liquid after centrifugation added to the contents of the second beaker. The second clean sediment is discarded. The bacterial suspension now in the second beaker is again centrifugated in the same way and a third portion of bacteria-free sediment separated and discarded. Frequently a fourth serial centrifugation is performed, always if the third sediment is of appreciable quantity. It will be noted that the method of separation is one of fractional sedimentation, differing somewhat from the procedure as practiced by Strasburger who centrifugates only twice in series, and discards the second sediment without washing it, and differing also from that of Steele who centrifugates once and then filters the suspension through muslin to remove the non-bacterial solid matter still remaining in it. We believe that our method gives more accurate results and that the final suspension contains less non-bacterial matter.

At all stages of the separation small portions of the dilute hydrochloric acid should

\*In case of uncertainty, microscopic examination should be resorted to. This is especially necessary before one has become familiar with the method.



be used for the washings, so that the final suspension shall not be too voluminous. Ordinarily it amounts to 150 to 300 c.c.

To the final bacterial suspension an equal volume of alcohol is added and the beaker set aside to concentrate. For this purpose Strasburger set the dish into a water bath at 40° C. We employ a bacteriological incubator regulated at a temperature of 50° C. in which the evaporation of the liquid is accomplished in about a week. When concentrated to about 50 c.c. the beaker is removed, about 200 c.c. of alcohol added, covered, and allowed to stand in the room 24 hours. At the end of this time the bacterial substance is generally settled so that most of the clear supernatant liquid can be directly siphoned off without loss of solid matter. The remainder is then transferred to two centrifuge tubes, centrifugated, and the remaining clear liquid pipetted off and discarded. The sediment is next treated with absolute alcohol, mixed with the alcohol and allowed to stand from  $\frac{1}{2}$  to 1 hour; then centrifugated and the alcohol removed. The tubes are now filled with ether, the sediment stirred up with a glass rod, the tubes corked and set aside for 24 hours. The cork is held in position by passing a rubber band over it and several times around the lower tapering end of the tube.

On the following day the ether is removed, the sediment washed with absolute alcohol, with aid of the centrifuge and by stirring with a glass rod, and then transferred to a weighed porcelain crucible by means of 95 per cent alcohol. The tube is carefully cleaned with a rubber-tipped rod and alcohol, so that all the sediment is finally in the crucible. With a little care and practice one can get all the sediment transferred to a small No. 00 crucible. Exceptionally it may happen that the crucible becomes full before the tube is clean, in which case the former is placed in the oven and allowed to dry down somewhat before adding the last washing. The crucible is next placed at 50° C. until the excess of liquid has evaporated and then dried at 102° for 48 hours, cooled in a dessicator, and weighed. It is then replaced at 102° C. and weighed again on each succeeding day until the weight is constant within 1 mg. From the data thus determined the percentage of dry bacterial substance in the moist feces and the percentage of bacterial substance in the dry feces are calculated. By applying the factors obtained by a series of such determinations to the total amounts of feces excreted over a considerable period, the average weight of dry bacterial substance excreted daily has been calculated.

#### NITROGEN.

The nitrogen in the bacterial residues was determined by the Kjeldahl method. This served as a most valuable check upon the purity of the bacterial residue. In general we rejected as unsatisfactory those Strasburger determinations the residues of which contained less than 10 per cent of nitrogen. Some residues contained as much as 12½ per cent of nitrogen. Undoubtedly there is considerable variation in the nitrogen content of different bacterial cells and of cells of the same species under different conditions of growth as has been clearly shown by Cramer.<sup>2</sup> We have found, however, that when the high-speed electric centrifuge was employed the nitrogen content fell below 10 per cent so rarely that technical error was always suspected when it did occur.

From the nitrogen factors thus obtained we have calculated the average daily bacterial nitrogen in the feces and the percentage of total fecal nitrogen represented by it. This ratio between bacterial nitrogen and total nitrogen of the feces would seem to be a fairly constant one under normal conditions when a uniform diet is being taken, and its determination in pathological conditions may prove of value. It can, of course,

be determined from a single stool and does not require the separate collection and weighing of the feces for definite periods, which is necessary for the estimation of the quantity of daily bacteria by the Strasburger method.

*Example.*—Strasburger determination—No. 252, Subject H, July 15, 1908.

(a) Dry substance.

	Fresh	Dry
(1) Crucible No. 8 + feces.....	10.3000 gm.	7.5234 gm.
Crucible No. 8.....	6.9561	6.9561
Feces.....	3.3439	0.5673
(2) Crucible No. 12 + feces.....	10.3965 gm.	7.6449 gm.
Crucible No. 12.....	7.0913	7.0913
Feces.....	3.3052	0.5536
Dry substance per gram feces = (1)	$\frac{0.5673}{3.3439}$	= 0.16965 gm.
(2)	$\frac{0.5536}{3.3052}$	= 0.16749

Average (1 and 2) = ..... 0.16857

(b) Dry bacteria.

(1) Centrifuge tube + feces.....	10.0206 gm.
Centrifuge tube.....	7.7316
Feces taken for separation of bacteria.....	2.2890
Crucible No. 14 + separated bacteria, dry.....	7.9516 gm.
Crucible No. 14.....	7.8362
Dry bacteria.....	0.1154
(2) Centrifuge tube + feces.....	9.2812 gm.
Centrifuge tube.....	7.4363
Feces taken for separation of bacteria.....	1.8449
Crucible No. 15 + separated bacteria, dry.....	7.0990 gm.
Crucible No. 15.....	7.0063
Dry bacteria.....	0.0927
Dry bacteria per gram feces = (1)	$\frac{0.1154}{2.2890} = 0.05042 \text{ gm.}$
(2)	$\frac{0.0927}{1.8449} = 0.05025$

Average (1 and 2) = 0.05033 gm.

(c) Dry bacterial substance in dry feces =  $\frac{0.05033}{0.16857}$  = 29.86 per cent.

(d) Nitrogen in bacterial residue.

- (1) Crucible No. 14 = 0.013419 gm. of nitrogen.  
 (2) Crucible No. 15 = 0.010823 " " "

Nitrogen in dry bacteria = (1)	$\frac{0.013419}{0.1154}$	= 11.63 per cent.
(2)	$\frac{0.010823}{0.0927}$	= 11.67 per cent.

Average (1 and 2) = 11.65 per cent.

- (e) Average dry bacterial substance excreted daily.  
 Average daily dry substance of feces of subject during this eight-day period = 20.508 gm.  
 Average daily dry bacterial substance =  $20.508 \times 29.86 = 6.13$  gm.
- (f) Ratio of bacterial nitrogen to total fecal nitrogen.  
 Average daily nitrogen of feces of subject at this period = 1.495 gm.  
 Average daily bacterial nitrogen = 11.65 per cent of 6.13 = 0.717 gm.  
 Bacterial in total nitrogen =  $\frac{0.717}{1.495} = 48.0$  per cent.

## DIFFERENTIAL COUNT.

In estimating the relative numbers of morphologically different bacteria of the feces, thin coverglass film preparations of the 1:100 suspension were employed. These were stained by a modified Gram's method,<sup>20</sup> thus adding to the different forms the further distinction afforded by this stain. The technic of the method is as follows:

1. Make a rather thin, even coverglass film preparation.
2. Dry in the air and fix in the flame.
3. Float the coverglass on anilin water gentian violet three minutes.
4. Drain off the excess of dye with filter paper, without washing.
5. Float on Lugol's solution two minutes.
6. Drain and dry the preparation between filter papers.
7. Decolorize in anilin-xylol by immersion for one-half minute.
8. Dry between filter papers.
9. Decolorize further in absolute methylic alcohol for about five seconds.
10. Wash in water.
11. Counterstain with a dilute aqueous solution of fuchsin for a few seconds.
12. Wash in water, blot, dry in the air, and mount in rectified balsam.

The anilin water gentian violet is freshly prepared by adding 0.5 c.c. saturated alcoholic solution of gentian violet to 10 c.c. of saturated aqueous solution of anilin. The Lugol's solution used contains 1 gm. iodine and 2 gm. potassium iodide in 300 c.c. distilled water. The anilin-xylol is made by mixing anilin, one part, with xylol, four parts. In the stained preparation five hundred bacteria are counted and classified according to form and color.

Considerable difficulty was experienced in accurately differentiating Gram negative and Gram positive forms and it cannot be doubted that bacteria of the same species were red in some preparations and blue in others, while it is equally certain that different parts of the same coverglass frequently showed these variations. Sometimes it was very difficult or even impossible to distinguish clearly between the bacteria and minute bits of other material present in the preparation. When we remember the large proportion of fecal bacteria already dead and disintegrating the difficulty of differential staining is not surprising. The procedure has however seemed to us to be of some value in the study of feces of adults.\* Its value in the case of infants is already well known.

## QUANTITY OF FECAL BACTERIA.

It has long been known that a considerable portion of the substance of the human feces consists of bacterial cells. Eberles, working in Escherich's laboratory, found an average of 33,000,000 bacteria per milligram feces, in a two-months-old, milk-fed

\* Cohendy, *Comptes rendus Soc. de Biol.*, 1906, 60, p. 415, gives a short notice of a similar study.

infant. Hellström<sup>7</sup> studied the fecal flora of the new-born, both by the microscopic and by the plate method. He found that the number of bacteria in the feces increased very rapidly after birth and by the end of the fourth day had become 40,000,000 bacterial cells per milligram feces, a result very close to that of Eberle. Cornelia de Lange<sup>4</sup> studied the fecal flora of infants, employing the counting method of Klein. In a series of cases, she found values from 42,000,000 to 932,000,000 cells per milligram dry substance, or from 11,000,000 to 298,000,000 bacteria per milligram of fresh feces, and concluded that the number of fecal bacteria in milk-fed infants is less than 1,000,000,000 per milligram fecal dry substance.

Klein<sup>11</sup> counted the bacteria in the feces of adults and in a series of fourteen examinations found from 20,162,000 to 165,614,000 bacteria per milligram, and an average of 58,800,000 per milligram fresh feces. The number of fecal bacteria excreted in 24 hours, he found to be, minimum,  $30 \times 10^{11}$ , maximum,  $248 \times 10^{11}$ , average  $88 \times 10^{11}$ . He estimated\* the average weight of the daily bacterial excretion as 293 mg., the bacterial portion of the dry substance as 0.13 per cent, and the bacterial nitrogen excreted daily in the feces as 4.39 mg. Strasburger<sup>23</sup> found by his gravimetric method that about one-third of the dry substance of normal adult human feces consists of bacterial substance. In a series of 16 cases, most of them pathological, he obtained values from 17.2 per cent to 68.4 per cent. The daily quantity of dry bacterial substance in this series varied from 2.6 gm. to 20.0 gm. Three of the subjects were normal individuals, and in these cases the daily bacterial dry substance (average of three days) was 11.8, 6.6, and 5.8 gm., giving an average of 8.1 gm. From this quantity he calculated the number of bacteria excreted daily to be  $128 \times 10^{12}$ , a number about 15 times as great as that of Klein. He did not enumerate the bacteria by the microscopic method. Strasburger mixed the bacterial residues from his several determinations and estimated the nitrogen content of the mixture in duplicate, obtaining 10.364 and 10.305 per cent of nitrogen. From this he concluded that more than half the fecal nitrogen is contained in the bacteria. Lissauer<sup>15</sup>, using Strasburger's procedure, determined the quantity of bacteria in 19 different stools from eight different persons. He found from 2.53 per cent to 13.54 per cent of the dry fecal substance made up of bacteria, with an average of 8.67 per cent, a value much lower than the result obtained by Strasburger. Steele<sup>22</sup> employed a modification of Strasburger's method. In three normal adults he found the bacterial substance to be 18.1, 21.1, and 18.2 per cent of the fecal dry substance, and the bacterial dry substance excreted daily, 7.44, 4.99, and 2.74 gm. or an average of 5.06 gm. per diem, comparatively close to the result obtained by Strasburger.

From this brief review of the more important literature it is evident that the results of the enumeration method do not agree closely with those of the gravimetric method, as the following extreme figures will indicate.

TABLE 1.

	Bacteria per Milligram Moist Feces	Daily Fecal Bacteria	Daily Bacterial Dry Substance Gm.	Bacteria in Fe- cal Dry Sub- stance—Per ct.	Daily Bacterial Nitrogen Gm.
Klein.....	58,800,000	$8.8 \times 10^{12}$	0.203	0.13	0.00439
Strasburger.....	.....	$128.0 \times 10^{12}$	8.100	33.00	0.83700

\* Klein<sup>12</sup> now regards these estimations as inaccurate and has repeated the calculations on a different basis, arriving at much higher results.

## EXPERIMENTAL RESULTS.

In Table 2, A to L, are given the data of the quantitative examination of 204 stools from 12 different subjects. The bacteria were counted by the Winterberg method and by the Klein method for each of these. The tables also show the results of the gravimetric determination of the bacteria by the method of Strasburger upon 141 of the 204 stools. Of these 141 examinations 42 of the first are not considered sufficiently accurate for comparative purposes. The last 99 gravimetric determinations were performed according to the method we have detailed above (page 129), are nearly all done in duplicate, and have the nitrogen determination made upon the bacterial residues. From these the determination of Subject D, May 28, should be excluded on account of the low nitrogen result, leaving 98 determinations which we regard as fairly representative of the results to be expected from this method.

The highest single count by the Winterberg method occurs in Subject C, Nov. 26, 1907,  $816 \times 10^6$  per milligram. Among the recent examinations the highest count occurs in Subject H, June 12, 1908,  $751 \times 10^6$  bacteria per milligram fresh feces. The lowest count by this method occurs in Subject B, Nov. 10, 1907,  $124 \times 10^6$  bacteria per milligram feces. The lowest counts among the recent examinations occur in Subject D, July 1,  $176 \times 10^6$  per milligram, and Subject L, May 25, 1908,  $220 \times 10^6$  per milligram. Of the 12 individuals, the highest average counts of all determinations by this method are in Subject C,  $490 \times 10^6$  per milligram, and Subject H,  $481 \times 10^6$  per milligram; the lowest average counts are in Subject B,  $275 \times 10^6$  per milligram, Subject K,  $280 \times 10^6$  per milligram, and Subject D,  $302 \times 10^6$  per milligram. The grand average of all 12 subjects by this method of counting is  $384 \times 10^6$  bacteria per milligram of fresh feces.

Enumeration by the Eberle-Klein method gave the highest single counts in Subject H, April 25, 1908,  $723 \times 10^6$  per milligram, and in Subject H, June 12, 1908,  $663 \times 10^6$  per milligram; the lowest single counts in Subject L, Jan. 20,  $149 \times 10^6$  per milligram, and Subject B, Dec. 9, 1907,  $163 \times 10^6$  per milligram. Of the 12 individuals, Subject H with  $469 \times 10^6$  per milligram and Subject C with  $432 \times 10^6$  per milligram show the highest average counts by this method. The lowest are in Subject K,  $293 \times 10^6$ , and Subject D,  $309 \times 10^6$  per milligram. The grand average of the 12 subjects by this counting method



is  $375 \times 10^6$  bacteria per milligram feces. The results of these two methods of counting correspond fairly well, the differences being less than 30 per cent in most instances. Much larger discrepancies occur, for example in Subject L, May 25, but these are not common, and as a rule the average of the two counts would appear to be reliable within 20 per cent.

The mean of the two counts has been used as the basis of comparison with the gravimetric results, for the calculation of the number of bacteria per gram of fecal dry substance, and for the estimation of the daily excretion of bacteria. The highest average mean value occurs in Subject H,  $475 \times 10^6$  per milligram, and the lowest in Subject K,  $287 \times 10^6$  per milligram. The grand average mean of the two counts is  $379 \times 10^6$  per milligram.

Of the 204 stools in which the bacteria were counted, the dry substance was determined in 148. For these the number of bacteria per gram dry fecal substance has been calculated and is recorded in Table 2, A to L. The lowest single value is in Subject D, Jan. 9,  $983 \times 10^9$  bacteria per gram fecal dry substance. The highest single value is in Subject C, Feb. 20,  $2642 \times 10^9$  bacteria per gram fecal dry substance. Subject D also shows the lowest average value of all examinations,  $1371 \times 10^9$  bacteria per gram dry feces, and Subject C the highest average value,  $1856 \times 10^9$  bacteria per gram dry feces. The grand average of all the examinations on the twelve individuals is  $1587 \times 10^9$  bacteria per gram dry feces.

The quantitative determination of the fecal bacteria by the Strasburger method yielded results which are expressed first as amount of bacterial dry substance per gram moist feces, and second as percentage bacterial dry substance in fecal dry substance. The first set of figures is comparable with the results of enumeration and the second set is comparable with the values obtained by Strasburger and by Steele. These percentages of bacterial substance in dry substance have also been employed to calculate the daily bacterial excretion (Table 4, A to L).

The greatest quantity of bacterial dry substance per gram moist feces in a single determination was obtained in Subject H, July 24, 1908, 0.132 gm. The next highest value occurred in the same subject June 12, 0.127 gm. The smallest quantity was found in Subject



L, May 25, 0.026 gm.; the next smallest in Subject D, July 17, 0.028 gm. Of the 12 individuals, Subject H gave the highest average value for all examinations, 0.097 gm. dry bacteria per gm. moist feces; Subjects D and K gave the lowest, 0.041 gm. in each case. The grand average for the 12 individuals is 0.070 gm. dry bacteria per gram fresh feces.

The duplicate gravimetric determinations agree fairly well as a rule and in some cases the agreement is very close. Large discrepancies are not rare, however, for example Subject I, April 29, and Subject B, May 1. These are probably due to definite error in technic rather than inherent in the method. The determination in duplicate and the estimation of the nitrogen in the bacterial residues checked them in such a way that the average results of the gravimetric method are probably more reliable, as a rule, than the mean of the two enumerations.

The percentage of dry bacterial substance in the fecal dry substance for a single determination was highest in Subject H, July 24, 42.53 per cent. Other high values were found in Subject H, June 12, 40.17 per cent, and in Subject F, May 7, 40.03 per cent. The lowest values were found in Subject D, July 17, 13.26 per cent, Subject L, May 25, 14.03 per cent, and Subject E, March 16, 14.53 per cent.

Of the 12 individuals Subject H showed the highest average bacterial substance in the dry fecal substance, 34.62 per cent (mean of nine determinations); Subject I followed with 32.06 per cent. The lowest average values were in Subject K, 20.08 per cent, and Subject D, 21.10 per cent. The grand average for the 12 individuals is 26.89 per cent bacterial substance in the dry fecal substance.

The determinations of the nitrogen in the bacterial residues were made by Mr. Gill in the analytical division of the Laboratory at the suggestion of Professor Grindley. Residues in which the nitrogen fell below 9.5 per cent were considered too impure to be used as a basis of calculation. Nearly all of them show more than 10 per cent of nitrogen, a few as much as 12 per cent. The grand average of all the nitrogen determinations on the satisfactory residues is 10.96 per cent.

A comparison of the quantitative determination of the fecal bacteria by the counting method with the results of the gravimetric method is of some interest. The results of the two methods correspond to a cer-

TABLE 2.  
FECAL BACTERIA. QUANTITATIVE DETERMINATIONS.  
SUBJECT A.

LABORATORY NO.	DATE	WT. OF STOOL Gm.	BACTERIA PER GRAM BY ENUMERATION $\times 10^6$				DRY BACTERIA PER GRAM BY STRASBURGER METHOD		DRY SUBSTANCE PER GRAM.		BACTERIA IN DRY FECES		PER CENT OF NITROGEN IN BACTERIA			
			Winterberg	Ratio	Klein	Mean	Ratio	1	2	Mean	No. per gram $\times 10^6$	Per cent by Wt.	1	2	Mean	
B 37	Nov. 12	63.2	266	..	406	..	336	..	.....	.....	.....	.....	.....	.....	.....	.....
B 60	Nov. 26	127.8	356	..	256	..	306	..	.....	.....	.....	.....	.....	.....	.....	.....
B 83	Dec. 10	115.4	371	..	344	..	358	..	.....	.....	.....	.....	.....	.....	.....	.....
B 110	Jan. 6	70.4	260	..	228	..	244	..	.....	.....	.....	.....	.....	.....	.....	.....
B 122	Jan. 22	80.5	377	34	302	27	330	30	0.112	.....	.....	.....	.....	.....	.....	.....
B 134	Feb. 6	118.7	380	32	317	27	348	26	0.118	.....	.....	.....	.....	.....	.....	.....
B 146	Feb. 24	126.6	363	27	332	25	348	26	0.134	0.137	0.272	1246	41.52	.....	.....	.....
B 158	Mar. 11	41.1	414	66	479	76	447	71	0.063	.....	.....	.....	.....	.....	.....	.....
B 170	Mar. 27	62.2	518	62	426	51	472	56	0.001	0.076	0.084	1368	24.22	9.00	10.80	10.35
B 182	Apr. 10	60.2	443	81	372	68	407	74	0.052	0.059	0.345	1304	18.07	12.49	11.26	11.88
B 194	Apr. 27	34.9	477	51	476	51	478	51	0.005	0.001	0.003	1345	26.26	.....	.....	.....
B 206	May 13	43.4	360	58	358	58	359	58	0.002	0.003	0.355	1026	17.81	.....	10.46	10.46
B 218	May 28	18.0	471	46	449	44	460	45	0.103	(0.131)	0.332	1386	35.34	11.55	(8.24)	11.55
B 230	June 15	60.4	534	45	545	40	539	45	0.113	0.124	0.338	1500	35.00	10.40	10.16	10.28
B 242	July 1	32.5	626	70	310	35	472	52	0.087	0.093	0.347	1300	25.88	10.30	10.12	10.21
B 254	July 17	63.3	375	101	310	86	347	94	0.038	0.036	0.244	1428	15.32	10.34	10.46	10.40
B 266	July 31	64.7	564	87	318	49	441	68	0.007	0.063	0.332	1332	19.65	.....	11.36	11.36
Average	.....	.....	421	67*	367	54*	394	60*	.....	.....	0.326*	1300	24.27*	.....	.....	10.81*
Minimum	.....	.....	.....	45*	.....	35*	.....	45*	.....	.....	.....	.....	.....	.....	.....	.....
Maximum	.....	.....	.....	101*	.....	86*	.....	94*	.....	.....	.....	.....	.....	.....	.....	.....

SUBJECT B.

B 33	Nov. 10	120.9	124	..	305	..	215	..	.....	.....	.....	.....	.....	.....	.....	.....	.....
B 50	Nov. 22	101.8	206	..	321	..	300	..	.....	.....	.....	.....	.....	.....	.....	.....	.....
B 79	Dec. 9	139.9	282	..	163	..	223	..	.....	.....	.....	.....	.....	.....	.....	.....	.....
B 102	Dec. 26	106.7	136	..	166	..	151	..	.....	.....	.....	.....	.....	.....	.....	.....	.....
B 114	Jan. 12	123.4	102	..	304	..	248	..	.....	.....	.....	.....	.....	.....	.....	.....	.....
B 126	Jan. 27	90.1	276	39	365	52	325	46	0.070	.....	0.070	1570	34.04	7.25	7.40	7.32	
B 138	Feb. 13	157.8	220	28	257	32	230	30	0.082	0.078	0.080	1300	28.47	7.25	7.40	7.32	
B 150	Mar. 2	44.6	248	46	320	59	284	53	0.055	0.053	0.100	1405	28.47	10.28	10.30	10.32	

SUBJECT C.

SUBJECT D.

\* Determinations previous to March 16 are not included in this calculation.

TABLE 2.—Continued  
SUBJECT D

LABORATORY No.	DATE	WT. OF STOOL Gm.	BACTERIA PER GRAM BY ENUMERATION $\times 10^6$					DRY BACTERIA PER GRAM BY STRASSBURGER METHOD				DRY SUBSTANCE PER GRAM		BACTERIA IN DRY FECES		PER CENT OF NITROGEN IN BACTERIA		
			Winterberg	Ratio	Klein	Ratio	Mean	Ratio	1	2	Mean	1	2	No. per gram $\times 10^6$	Per cent by Wt.	1	2	Mean
B 80	Dec. 10	128.9	272	..	307	..	289	..	..	..	..	..	..	..	..	..	..	
B 99	Dec. 23	20.0	336	..	439	..	387	..	..	..	..	..	..	..	..	..	..	
B 112	Jan. 9	139.0	220	32	236	35	228	34	0.068	..	0.232	083	20.48	..	..	..	..	
B 123	Jan. 25	168.6	284	23	240	20	262	22	0.121	..	0.264	902	45.88	..	..	..	..	
B 135	Feb. 10	40.0	300	33	276	30	288	31	0.092	..	0.205	1308	44.77	9.00	..	9.00	..	
B 147	Feb. 27	64.8	276	45	270	44	270	44	0.049	0.002	0.240	1125	25.01	10.30	10.43	10.37	..	
B 157	Mar. 11	73.7	316	72	389	88	352	80	0.047	0.041	0.249	1408	17.52	10.94	..	10.94	..	
B 169	Mar. 27	123.8	380	78	264	54	322	66	0.052	0.047	0.202	1594	24.28	12.26	11.76	12.01	..	
B 181	Apr. 10	81.7	284	65	332	75	308	70	0.045	0.043	0.200	1532	21.81	12.50	12.67	12.58	..	
B 205	Apr. 27	235.6	224	53	280	67	252	60	0.040	0.035	0.103	1490	23.35	10.92	11.20	11.10	..	
B 205	May 13	157.3	272	57	385	80	328	68	0.061	0.036	0.164	1624	24.00	10.40	10.82	10.61	..	
B 217	May 28	20.6	356	(52†)	321	(47†)	339	(49†)	0.066	0.073	0.069†	1662	34.95†	8.96	7.95	8.45†	..	
B 220	June 15	45.0	276	59	254	54	265	56	0.037	0.038	0.187	1417	25.27	11.14	11.23	11.18	..	
B 241	July 1	96.1	176	50	210	60	103	55	0.037	0.034	0.173	1135	20.75	11.32	9.44	10.38	..	
B 253	July 17	22.0	288	103	325	116	307	110	0.027	0.029	0.224	1471	13.26	11.73	11.14	11.43	..	
B 265	July 31	171.7	288	85	320	94	304	89	0.032	0.036	0.213	1448	16.08	11.49	11.46	11.47	..	
Average.....			302	69*	309	75*	305	72*	0.041*	0.041*	0.105*	1371	21.10*	..	..	..	11.35*	
Minimum.....			50*	50*	..	54*	..	55*	..	..	..	..	..	..	..	..	..	
Maximum.....			103*	103*	..	116*	..	110*	..	..	..	..	..	..	..	..	..	

SUBJECT E.

B 41	Nov. 14	40.9	248	..	260	..	254	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	
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B185	Apr. 16	32.5	468	51	413	45	441	48	0.095	0.088	0.092	0.285	0.288	0.286	1542	32.02	11.17	11.46	11.31
B197	May 18	40.5	533	68	405	63	514	66	0.085	0.071	0.078	0.327	0.321	0.324	1586	24.04	10.36	10.89	10.61
B209	May 18	18.5	058	89	368	59	513	69	0.074	0.071	0.074	0.311	0.311	0.311	1504	21.56	10.13	10.37	10.13
B221	June 3	138.0	333	46	354	47	468	47	0.077	0.070	0.073	0.231	0.233	0.232	1478	21.71	11.30	11.37	11.38
B233	June 10	53.8	450	63	450	67	468	65	0.071	0.072	0.072	0.289	0.272	0.281	1665	25.50	11.68	11.51	11.60
B245	July 6	33.5	397	55	340	48	373	52	0.071	0.073	0.072	0.240	0.231	0.236	1583	30.42	11.17	11.64	11.40
B257	July 22	63.9	058	87	481	63	569	75	0.076	0.076	0.076	0.201	0.284	0.288	1076	26.46	10.80	11.07	10.80
Average	Minimum	446	446	70*	403	62*	424	66*	0.071*	0.071*	0.071*	0.201	0.284	0.272*	1652	25.79*	11.17	11.46	10.94*
Maximum	.....	.....	.....	46*	415*	113*	..	107*	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....

SUBJECT F.

B 38	Nov. 13	48.8	400	..	393	..	396	..	0.139	0.088	0.139	0.318	0.318	0.318	1208	39.77	9.18	10.62	9.18
B 61	Nov. 26	44.0	364	..	406	..	430	..	0.155	0.074	0.155	0.388	0.388	0.388	1200	34.42	12.86	10.62	10.62
B 84	Dec. 12	25.9	328	..	393	..	360	..	0.042	0.074	0.074	0.214	0.214	0.214	1220	34.42	10.80	10.73	10.81
B103	Dec. 28	21.5	404	..	455	..	459	..	0.122	0.112	0.117	0.311	0.311	0.311	1406	37.71	10.80	10.73	10.81
B115	Jan. 14	9.0	464	..	466	..	405	..	0.069	0.069	0.071	0.252	0.254	0.253	1838	28.12	11.44	12.33	11.88
B127	Jan. 20	39.2	436	31	331	24	384	28	0.073	0.068	0.086	0.206	0.204	0.205	1688	29.16	11.20	10.55	10.88
B139	Feb. 17	69.8	522	34	415	27	469	33	0.084	0.116	0.122	0.366	0.303	0.304	1566	40.03	10.39	10.39	10.39
B153	Mar. 6	79.4	232	31	294	40	263	36	0.072	0.078	0.072	0.253	0.259	0.255	1600	28.14	10.23	10.23	10.23
B165	Mar. 23	13.9	460	39	453	39	456	39	0.073	0.072	0.072	0.287	0.284	0.286	1647	25.22	10.42	10.08	10.25
B177	Apr. 6	32.5	444	63	486	68	495	66	0.072	0.070	0.071	0.304	0.305	0.304	1391	23.42	11.16	11.35	11.25
B189	Apr. 22	42.7	476	55	521	61	498	58	0.070	0.069	0.069	0.250	0.248	0.240	1079	27.83	11.30	11.25	11.27
B201	May 7	22.8	492	40	461	38	476	39	0.042	0.040	0.041	0.206	0.211	0.200	1904	19.58	10.72	11.42	11.07
B213	May 23	54.5	412	57	404	56	468	57	0.066	0.078	0.072	0.253	0.259	0.255	1600	28.14	10.23	10.23	10.23
B225	June 8	57.9	444	62	511	71	477	66	0.073	0.072	0.072	0.287	0.284	0.286	1647	25.22	10.42	10.08	10.25
B237	June 25	23.1	460	65	386	54	423	60	0.070	0.070	0.071	0.304	0.305	0.304	1391	23.42	11.16	11.35	11.25
B249	July 13	35.3	472	68	365	53	418	61	0.070	0.069	0.069	0.250	0.248	0.240	1079	27.83	11.30	11.25	11.27
B261	July 27	83.4	340	83	450	111	398	97	0.042	0.040	0.041	0.206	0.211	0.200	1904	19.58	10.72	11.42	11.07
Average	Minimum	401	401	59*	405	61*	403	66*	0.080*	0.080*	0.080*	0.206	0.211	0.200	1535	28.80*	11.17	11.42	10.80*
Maximum	.....	.....	.....	83*	..	111*	..	97*	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....

SUBJECT G.

B 42	Nov. 15	137.5	224	..	193	..	208	..	0.071	0.071	0.071	0.201	0.284	0.272*	1652	25.79*	11.17	11.46	10.94*
B 55	Nov. 20	380	380	..	302	..	341	..	0.071	0.071	0.071	0.201	0.284	0.272*	1652	25.79*	11.17	11.46	10.94*
B 88	Dec. 14	128.4	166	..	192	..	179	..	0.071	0.071	0.071	0.201	0.284	0.272*	1652	25.79*	11.17	11.46	10.94*

\* Determinations previous to March 16 are not included in this calculation.

† Not included in the average calculation on account of low nitrogen.



TABLE 2.—Continued  
SUBJECT G

LABORATORY No.	DATE	WT. OF STOOL Gm.	BACTERIA PER GRAM BY ENUMERATION $\times 10^9$				DRY BACTERIA PER GRAM BY STRASBURGER METHOD			DRY SUBSTANCE PER GRAM			BACTERIA IN DRY FECES		PER CENT OF NITROGEN IN BACTERIA	
			Winterberg	Ratio	Klein	Ratio	Mean	Ratio	1	2	Mean	No. per gram $\times 10^9$	Per cent by Wt.	1	2	Mean
B107	Jan. 2	114.3	268	..	356	..	327	..	0.140	0.076	0.076	1632	55.50	.....	.....	.....
B119	Feb. 3	20.9	260	10	250	10	200	24	0.157	0.068	0.068	1243	51.72	.....	.....	.....
B131	Feb. 3	38.8	378	24	379	24	370	24	0.157	0.105	0.105	1786	43.44	10.61	9.81	.....
B143	Mar. 4	66.9	593	46	475	37	534	44	0.111	0.143	0.200	1570	36.06	.....	0.93	.....
B152	Mar. 4	41.0	518	46	441	39	480	43	0.111	0.114	0.304	1593	34.37	10.55	.....	.....
B174	Mar. 19	73.4	527	53	351	35	439	44	0.064	0.107	0.203	1593	34.37	10.55	.....	.....
B178	Apr. 3	31.4	527	..	421	..	474	..	.....	.....	.....	.....	.....	.....	.....	.....
B188	Apr. 20	50.8	466	65	439	58	468	62	0.076	0.076	0.284	1677	27.23	10.63	10.05	.....
B200	May 4	30.5	468	42	483	49	446	46	0.068	0.100	0.207	1522	33.02	10.72	10.72	.....
B212	May 20	30.5	600	57	393	37	497	47	0.100	0.105	0.204	1602	34.99	11.18	10.27	.....
B224	June 5	220.5	285	77	294	79	200	78	0.037	0.036	0.154	1812	22.81	11.69	11.67	.....
B236	June 22	220.5	362	71	450	83	416	77	0.052	0.055	0.054	2133	28.85	11.78	(14.86)	.....
B248	July 6	97.6	526	60	440	50	483	55	0.084	0.088	0.309	1548	28.09	10.62	10.64	.....
B264	July 29	40.0	688	78	478	54	583	66	0.087	0.089	0.320	1845	27.82	10.66	11.04	.....
Average	.....	.....	427	63*	373	56*	400	50*	.....	.....	0.260*	1612	27.15*	.....	.....	10.08*
Minimum	.....	.....	.....	42*	.....	35*	.....	44*	.....	.....	.....	.....	.....	.....	.....	.....
Maximum	.....	.....	.....	78*	.....	83*	.....	78*	.....	.....	.....	.....	.....	.....	.....	.....

SUBJECT H

LABORATORY No.	DATE	WT. OF STOOL Gm.	BACTERIA PER GRAM BY ENUMERATION $\times 10^9$				DRY BACTERIA PER GRAM BY STRASBURGER METHOD			DRY SUBSTANCE PER GRAM			BACTERIA IN DRY FECES		PER CENT OF NITROGEN IN BACTERIA	
			Winterberg	Ratio	Klein	Ratio	Mean	Ratio	1	2	Mean	No. per gram $\times 10^9$	Per cent by Wt.	1	2	Mean
B 48	Nov. 18	79.0	309	..	355	..	377	..	.....	.....	.....	.....	.....	.....	.....	.....
B 71	Dec. 3	31.8	524	..	546	..	535	..	.....	.....	.....	.....	.....	.....	.....	.....
B 04	Dec. 19	102.1	384	..	344	..	364	..	.....	.....	.....	.....	.....	.....	.....	.....
B109	Jan. 6	53.8	456	..	360	..	412	..	.....	.....	.....	.....	.....	.....	.....	.....
B121	Jan. 22	38.2	420	26	347	22	383	24	0.150	0.150	0.101	2157	48.03	.....	.....	.....
B133	Feb. 6	21.0	396	38	332	32	364	35	0.103	0.103	0.230	1157	54.92	.....	.....	.....
B145	Feb. 24	70.7	404	27	497	33	451	30	0.150	0.150	0.274	1646	54.71	8.89	7.81	8.39
B156	Mar. 10	27.7	517	49	518	49	517	49	0.100	0.100	0.279	1853	37.51	11.37	11.08	11.23
B168	Mar. 25	155.9	421	44	484	50	453	47	0.088	0.104	0.096	1701	37.95	11.44	11.49	11.47
B180	Apr. 8	63.5	532	53	468	47	500	50	0.099	0.101	0.100	1543	30.74	11.01	11.08	11.12
B192	Apr. 25	79.9	570	48	723	61	647	54	0.114	0.123	0.304	2107	38.64	11.12	(6.04)	.....
B204	May 11	22.1	444	46	468	49	456	48	0.081	0.110	0.316	1466	30.70	10.90	10.57	10.73
B216	May 25	80.9	439	55	584	73	512	64	0.075	0.084	0.268	1896	29.44	11.34	11.17	.....



	SUBJECT I															
	Average	Minimum	Maximum	June 12	June 20	June 26	July 1	July 5	July 12	July 18	July 24	July 30	Aug. 5	Aug. 12	Aug. 19	Aug. 26
B228	481	455	509	59	663	52	707	56	0.126	0.120	0.127	0.320	0.314	0.317	2230	40.17
B240	320	320	320	75	513	68	536	71	0.082	0.082	0.075	0.242	0.237	0.239	2243	31.51
B252	320	320	320	64	324	65	322	64	0.050	0.050	0.050	0.170	0.168	0.169	1005	29.86
B260	647	647	647	49	432	33	540	41	0.134	0.131	0.132	0.310	0.312	0.311	1736	42.53
Average	481	455	509	55*	460	55*	475	55*	0.097*	0.097*	0.097*	0.278*	0.278*	0.278*	1808	34.62*
Minimum	455	455	455	44*	324	33*	322	41*	0.050	0.050	0.050	0.170	0.168	0.169	1005	29.86
Maximum	647	647	647	75*	513	75*	540	71*	0.134	0.131	0.132	0.310	0.312	0.311	1736	42.53
SUBJECT J																
	SUBJECT J															
	Average	Minimum	Maximum	Nov. 11	Nov. 18	Nov. 25	Dec. 2	Dec. 9	Dec. 16	Dec. 23	Dec. 30	Jan. 6	Jan. 13	Jan. 20	Jan. 27	Feb. 3
B 32	260	260	260	241	251	251	251	251	251	251	251	251	251	251	251	251
B 58	304	304	304	303	348	348	348	348	348	348	348	348	348	348	348	348
B 75	248	248	248	333	200	200	200	200	200	200	200	200	200	200	200	200
B101	331	331	331	250	201	201	201	201	201	201	201	201	201	201	201	201
B113	321	321	321	348	335	335	335	335	335	335	335	335	335	335	335	335
B125	314	314	314	260	31	31	31	31	31	31	31	31	31	31	31	31
B137	454	454	454	542	44	44	44	44	44	44	44	44	44	44	44	44
B165	206	206	206	268	58	58	58	58	58	58	58	58	58	58	58	58
B172	416	416	416	468	442	442	442	442	442	442	442	442	442	442	442	442
B184	308	308	308	400	52	52	52	52	52	52	52	52	52	52	52	52
B196	480	480	480	434	58	58	58	58	58	58	58	58	58	58	58	58
B208	424	424	424	40	533	58	478	61	0.075	0.040	0.075	0.312	0.312	0.312	1405	24.00
B220	302	302	302	403	45	45	45	45	0.090	0.093	0.092	0.248	0.240	0.241	1935	37.20
B232	480	480	480	595	402	402	402	402	0.089	0.090	0.090	0.252	0.250	0.251	1582	35.78
B241	588	588	588	75	612	82	612	78	0.081	0.076	0.078	0.260	0.278	0.272	2250	28.80
B250	584	584	584	76	568	68	576	69	0.085	0.082	0.083	0.280	0.258	0.270	2133	30.82
Average	393	393	393	55*	412	61*	402	58*	0.085*	0.085*	0.085*	0.268*	0.268*	0.268*	1727	32.06*
Minimum	393	393	393	33*	324	33*	322	43*	0.050	0.050	0.050	0.170	0.168	0.169	1005	29.86
Maximum	584	584	584	75*	513	75*	540	71*	0.134	0.131	0.132	0.310	0.312	0.311	1736	42.53
SUBJECT K																
	SUBJECT K															
	Average	Minimum	Maximum	Nov. 6	Nov. 13	Nov. 20	Nov. 27	Dec. 4	Dec. 11	Dec. 18	Dec. 25	Jan. 1	Jan. 8	Jan. 15	Jan. 22	Jan. 29
B 31	131	131	131	184	158	158	158	158	158	158	158	158	158	158	158	158
B 54	207	207	207	347	322	322	322	322	322	322	322	322	322	322	322	322
B 77	284	284	284	274	279	279	279	279	279	279	279	279	279	279	279	279
B100	200	200	200	245	208	208	208	208	208	208	208	208	208	208	208	208
B111	250	250	250	346	44	44	44	44	44	44	44	44	44	44	44	44
B124	382	382	382	237	18	18	18	18	18	18	18	18	18	18	18	18
B136	210	210	210	30	230	32	220	31	0.071	0.132	0.132	0.272	0.272	0.272	1006	45.35
B148	380	380	380	383	20	386	20	386	0.071	0.132	0.132	0.272	0.272	0.272	1128	48.18
B159	347	347	347	380	48	304	46	304	0.081	0.133	0.133	0.286	0.277	0.282	1317	45.47
Average	247	247	247	247	247	247	247	247	0.077	0.077	0.077	0.277	0.277	0.277	1201	28.05
Minimum	247	247	247	247	247	247	247	247	0.077	0.077	0.077	0.277	0.277	0.277	1201	28.05
Maximum	347	347	347	380	48	304	46	304	0.081	0.133	0.133	0.286	0.277	0.282	1317	45.47

\* Determinations previous to March 16 are not included in this calculation.

TABLE 2.—Continued  
SUBJECT J

[illegible]

SUBJECT K

[illegible]

## SUBJECT L

[illegible]

\* Determinations previous to March 16 are not included in this calculation.

tain extent. After each column of enumeration data in Table 2 has been inserted a column of figures headed "ratio," in which the relation between the two values is expressed. The figure given in this column multiplied by  $10^{11}$  represents the number of bacterial cells according to that particular count, corresponding to one gram of bacterial dry substance according to the gravimetric analysis of the same sample of feces. Thus in Table 2, Subject A, May 13,  $359 \times 10^9$  bacteria per gram moist feces correspond to 0.062 gm. of bacterial dry substance per gram moist feces. At this rate  $58 \times 10^{11}$  bacteria would yield one gram bacterial dry substance. Considering the mean data from March 16 to July 31 only, this ratio or factor is seen to vary from 116 in Subject L, May 25, and 110 in Subject D, July 17, to 37 in Subject C, March 23, 39 in Subject F, March 23, and 40 in Subject J, March 30. All intermediate values are also represented. Of the 12 individuals Subject K shows the highest average value for this ratio, namely, 77; Subject J shows the lowest, 53. The grand average of all the examinations is 64. According to these figures it would appear that in one case three times as many fecal bacteria are required to yield a gram of dry substance as in another instance, while the average lies about midway between the two extremes. The discrepancy is considerable but appears quite insignificant in comparison with the disagreement of the results of Klein and of Strasburger previously mentioned (p. 134).

In attempting to account for this variation, experimental error is the first factor which occurs to one. This certainly applies to some instances at least, as for example in Subject L, May 25, where the two counts do not agree and the gravimetric duplicates show a variation of 20 per cent. This seems not to be true in every case, however. The differences between the average ratio for the different individuals can hardly be explained in this way.

In our comparisons between the gravimetric and the counting methods we have assumed that the water content of the bacterial cells in the fecal suspension is constant. This, as a matter of fact, is not the case, as Cramer<sup>1</sup> and Lyons<sup>16</sup> have shown that bacteria do not possess a typical water content, but that it varies according to the conditions of growth, such as for example, the kind of medium<sup>2</sup> and age of the culture. Cramer<sup>3</sup> found that a culture of a single species shows

wide variations in this respect. The bacteria of the feces include many species; there is considerable variation in the size of the various cells present and a very large majority of them are dead before the stool is passed. Spores are at times present in considerable numbers and these contain much more dry substance than vegetative cells.<sup>1</sup> One should therefore expect considerable variation in the chemical composition of the mixed fecal flora, especially in its dry-substance content. This variation in the dry-substance content of the bacterial cell is an important factor in accounting for the variation in the ratio between the two quantitative methods. By reference to Table 2, it will be seen that Subjects K, B, and D, the three subjects showing the lowest average counts, also show the smallest quantity of bacterial dry substance, the least percentage of dry substance in the feces, the least percentage of bacterial substance in the fecal dry substance, and **the highest enumeration-weight ratios**, namely, 77, 73, and 72. On the other hand, Subjects H, C, and I show high values for the counts and the gravimetric results, and the enumeration-weight ratio for each of these is low, 55, 58, and 58 respectively. Subject J with the lowest ratio is an exception to this general rule. In each individual also it will be seen that, as a general rule, an increase in the quantity of bacteria and an increase in the dry substance of the feces, is associated with a decrease in the enumeration-weight ratio of the bacteria. It would seem proper therefore to conclude that these various factors are to a certain extent associated with each other as cause and effect or as effects of the same cause.

Table 3 gives the mean of all determinations and the maximum and minimum single determinations of Table 2.

TABLE 3.  
SUMMARY OF SINGLE EXAMINATIONS.

	BACTERIA COUNTED		DRY BACTERIAL SUBSTANCE		DRY SUBSTANCE IN FECES	ENUMERATION-WEIGHT RATIO
	Per Gram Fresh Feces	Per Gram Dry Feces	Per Gram Fresh Feces Grams	In Fecal Dry Substance Per cent	Per cent	
Mean.....	$375 \times 10^9$	$1587 \times 10^9$	0.070	26.80	25.3	$64 \times 10^{11}:1$
Maximum.....	$816 \times 10^9$	$2642 \times 10^9$	0.132	42.53	35.4	$116 \times 10^{11}:1$
Minimum.....	$124 \times 10^9$	$983 \times 10^9$	0.026	14.03	16.1	$37 \times 10^{11}:1$

As has been said, the nitrogen in the dry bacterial residues was

found to be from 10 to 12 per cent, with an average of 10.96 per cent. These figures agree with those of Strasburger.<sup>23</sup> Cramer,<sup>2</sup> in his analysis of various bacteria grown in pure culture on agar, found 10 to 13 per cent of nitrogen in the dry substance. Attention should, however, be called to the circumstance that his percentages were calculated on the total dry bacterial substance, while ours, and also those of Strasburger, are calculated upon the residue after extraction with alcohol and ether. When Cramer's results are calculated on extracted residue the nitrogen values become 10 to 15 per cent greater. Although it is not impossible that the alcohol and ether extraction removes nitrogenous bodies, yet we should expect the net result to be an increase in the nitrogen percentage of the residue. In pure cultures of *B. coli* grown upon agar, Leach<sup>14</sup> found 10.65 per cent nitrogen in an air-dried sample not at constant weight. In another determination in which 173 gm. of air-dried cell substance was analyzed, she obtained 11 per cent of nitrogen. The cell substance employed for these analyses was the residue after extraction with alcohol and ether in each case.

We have performed a few experiments in a small way to determine, upon pure cultures, the number of bacteria required to yield a gram of dry bacterial substance extracted as in Strasburger's method, and to determine the nitrogen content of such a residue. For this purpose we used pure cultures of *B. coli* grown upon agar in large flat bottles at 37° C. After from five to eight days the bacterial growth was carefully washed off with a small amount of 0.8 per cent salt solution and obtained in a concentrated suspension. This was thoroughly mixed. Dilute suspensions were then prepared and the number of bacteria estimated by the same methods as have been employed in counting the fecal bacteria. In addition to this, portions (4 to 5 c.c.) of the concentrated suspension were accurately weighed in centrifuge tubes and suspended in 0.5 per cent hydrochloric acid, centrifugated, extracted, and dried, precisely as in the gravimetric method for separation of bacteria from feces. In three experiments of this kind, the relation between the two quantitative determinations agreed fairly well. In the first experiment the flask cultures were five days old and here  $55 \times 10^{11}$  bacteria by the counting methods corresponded to a gram of bacterial dry substance obtained by the gravimetric method. In the second experiment, in which the cultures were again five days old, the ratio was  $46 \times 10^{11}$  by count to a gram dry substance, and in the third experiment, with cultures eight days old,  $57 \times 10^{11}$  bacteria by count were found to be equivalent to a gram of the bacterial dry substance obtained by the gravimetric method. The nitrogen in these bacterial residues was 11.52 per cent in the first, 11.71 per cent in the second, and 11.80 per cent in the third experiment. It is thus evident that these methods yield, in their application to pure cultures of bacteria, results somewhat similar to those obtained upon bacteria of the feces. This we regard as important evidence of their reliability.



## AVERAGE DAILY EXCRETION OF BACTERIA.

The average daily excretion of bacteria, of bacterial nitrogen, and the percentage of fecal nitrogen represented by the latter, were calculated for each subject, by applying the values obtained in the bacteriological examination of a single stool to the average daily quantity of feces, of fecal dry substance, and of nitrogen, for the eight-day period in which the bacteriological examination occurred. The principal objection to his calculation lies in the circumstance that the stool examined was not necessarily a fair sample of the feces of that period; but in a series of examinations, errors introduced by this chance would tend to equalize each other. In one respect, however, the stools used for the bacteriological analyses were peculiar. For this special work the first stool passed after 6 A. M. was employed, so that in general, where the individual passed more than one stool a day, the feces employed for the bacteriological examination contained less water than the average. The error resulting from this cause would not seem to be very serious. It probably tends to render the values for the bacteria too high rather than too low.

Table 4, A to L, gives the data of these calculations. The daily number of fecal bacteria has been calculated in two ways; first, on the basis of the weight of the fresh feces, and second, on the basis of dry substance. Inasmuch as the number of bacteria per milligram varies with the dry substance in feces to a certain extent, the latter set of figures is the more accurate. Calculated on the basis of moist feces, the highest values, daily fecal bacteria by enumeration, occur in Subject I, July 3,  $64 \times 10^{12}$ , and Subject H, January 6,  $63 \times 10^{12}$ . In each of these instances the individual suffered from a rather marked diarrhea during the eight-day period concerned, serving to increase the amount of feces considerably above the average for that individual. For that reason these two estimates are unusual if not really abnormal. Subject H, April 25, shows a daily excretion of  $60 \times 10^{12}$  bacteria and on June 12,  $58 \times 10^{12}$  bacteria. The lowest values occur in Subject B, June 3,  $16 \times 10^{12}$  bacteria per day, Subject J, July 20,  $17 \times 10^{12}$  bacteria, and Subject B, April 16,  $18 \times 10^{12}$  bacteria. Of the 12 individuals, Subject H shows the highest average daily number of bacteria for the entire period of observation, namely  $45 \times 10^{12}$ . Subject L has an average of  $41 \times 10^{12}$ ; Subject D,  $40 \times 10^{12}$ . The lowest value is

TABLE 4  
DAILY FECAL BACTERIA AND BACTERIAL NITROGEN.  
SUBJECT A.

LABORATORY No.	PERIOD	AVERAGE DAILY FECES	AVERAGE DAILY SUBSTANCE	AVERAGE DAILY NITROGEN	DATE BACT. EXAM.	NUMBER OF BACTERIA				GRAVIMETRIC DETERMINATION				
						Calculated on Moist Feces		Calculated on Dry Feces		Dry Bacteria in Dry Substance Per cent	Daily Dry Bacteria Gm.	Daily Bacterial Nitrogen Gm.	Bacterial N. in Total Fecal N. Per cent	
						Per Gram. $\times 10^9$	Daily $\times 10^9$	Per Gram. $\times 10^9$	Daily $\times 10^9$					
B110	Jan. 4-11	112.85	27.760	1.636	Jan. 6	244	27,535	1,246	31,484	41.5	10.40	.....	.....	.....
B122	Jan. 20-27	106.45	25.268	1.605	Jan. 22	330	36,087	1,240	31,484	41.5	10.40	.....	.....	.....
B134	Feb. 5-12	89.48	25.015	1.479	Feb. 6	348	31,137	1,338	34,074	45.3	11.74	.....	.....	.....
B146	Feb. 21-28	70.06	20.028	1.141	Feb. 24	348	24,382	1,201	25,255	40.1	9.83	0.738	64.6	.....
B158	Mar. 8-15	78.24	21.166	1.170	Mar. 11	447	34,972	1,087	42,957	28.1	5.95	0.446	38.1	.....
B170	Mar. 24-31	52.65	16.659	0.915	Mar. 27	472	24,851	1,368	22,662	24.2	4.93	0.410	45.8	.....
B182	Apr. 9-16	52.15	15.372	0.814	Apr. 10	497	21,225	1,304	21,420	19.0	2.02	0.348	42.8	.....
B104	A. 25-M. 2	62.39	10.014	0.980	Apr. 27	476	20,697	1,345	25,574	26.3	5.00	0.540	54.6	.....
B206	May 11-18	60.28	18.654	1.032	May 13	359	21,639	1,026	19,130	17.8	3.32	0.340	33.8	.....
B218	M. 27-J. 3	70.08	19.259	1.081	May 28	466	32,235	1,386	26,681	35.3	6.80	0.673	62.2	.....
B230	June 12-19	50.34	16.130	0.909	June 15	539	27,132	1,509	25,647	35.0	5.95	0.582	64.0	.....
B242	J. 28-J. 5	66.21	23.135	1.408	July 1	472	45,413	1,366	31,404	25.0	5.00	0.611	33.2	.....
B254	July 14-21	68.80	19.253	1.125	July 17	347	23,874	1,428	27,493	15.3	2.95	0.306	27.2	.....
B266	July 22-29	62.33	19.559	1.172	July 31	441	27,485	1,339	26,177	19.0	3.83	0.437	37.3	.....
Average.....	.....	.....	.....	.....	.....	.....	28,172	.....	27,672	.....	4.50*	0.474*	44.5*	.....

SUBJECT B.

LABORATORY No.	PERIOD	AVERAGE DAILY FECES	AVERAGE DAILY SUBSTANCE	AVERAGE DAILY NITROGEN	DATE BACT. EXAM.	NUMBER OF BACTERIA				GRAVIMETRIC DETERMINATION				
						Calculated on Moist Feces		Calculated on Dry Feces		Dry Bacteria in Dry Substance Per cent	Daily Dry Bacteria Gm.	Daily Bacterial Nitrogen Gm.	Bacterial N. in Total Fecal N. Per cent	
						Per Gram. $\times 10^9$	Daily $\times 10^9$	Per Gram. $\times 10^9$	Daily $\times 10^9$					
B114	Jan. 12-19	79.34	14.005	0.822	Jan. 12	248	33,651	1,570	30,496	34.0	6.58	.....	.....	.....
B126	Jan. 20-27	112.72	18.023	0.969	Jan. 27	325	30,871	1,300	26,303	46.3	8.76	0.640	57.5	.....
B138	Feb. 13-20	71.14	15.508	1.111	Feb. 13	239	26,941	1,405	26,310	28.4	4.43	0.456	47.2	.....
B150	F. 20-M. 7	101.35	17.221	0.905	Mar. 2	284	27,587	1,704	30,804	26.6	4.38	0.481	49.0	.....
B162	Mar. 16-23	83.97	17.252	0.922	Mar. 16	296	30,000	1,034	28,190	19.6	1.87	0.194	38.2	.....
B174	Apr. 1-8	43.07	0.533	0.590	Apr. 16	416	18,294	1,496	14,261	20.0	3.02	0.320	39.1	.....
B186	Apr. 9-16	83.71	15.194	0.843	Apr. 16	379	20,340	1,458	22,922	19.6	2.66	0.303	33.8	.....
B198	A. 25-M. 2	77.44	13.504	0.806	May 18	260	25,341	1,600	21,792	27.1	2.81	0.334	61.9	.....
B210	May 11-18	53.61	10.359	0.540	June 3	298	15,977	1,035	20,945	23.8	4.73	0.530	51.2	.....
B222	M. 27-J. 3	53.61	10.359	0.540	June 3	298	15,977	1,035	20,945	23.8	4.73	0.530	51.2	.....
B234	June 12-19	97.89	19.878	1.054	June 19	380	37,197	1,959	38,941	23.8	4.73	0.530	51.2	.....

B246 B258	July 6-13	103.20	10.006	1.009	July 6	425	43,860	1,683	32,130	27.8	5.31	0.589	58.4
	July 22-29	84.54	10.592	0.871	July 22	260	22,741	1,503	24,038	21.3	3.53	0.371	42.6
Average.....	.....	.....	.....	.....	.....	.....	28,050	.....	26,007	.....	3.56*	0.393*	46.0*

SUBJECT C.													
B104	D. 27-J. 3	80.86	21.216	1.304	Dec. 28	350	29,030	.....	.....	.....	.....	.....	.....
	Jan. 12-19	18.061	15.318	1.033	Jan. 14	405	27,870	.....	.....	.....	.....	.....	.....
B116	J. 28-F. 4	78.70	10.326	1.258	Jan. 20	402	31,637	.....	.....	.....	.....	.....	.....
B128	Feb. 13-20	Feces were not collected for this period											
B140	.....	.....	.....	.....	.....	457	.....	2,642-	.....	.....	.....	.....	.....
B154	F. 20-M. 7	76.48	16.587	1.159	Mar. 6	463	35,408	2,303	38,200	31.4	5.21	0.578	40.9
B166	Mar. 16-23	65.71	15.318	1.033	Mar. 23	436	28,651	1,434	21,066	38.8	5.04	0.672	65.0
B178	Apr. 1-8	68.65	16.880	1.100	Apr. 6	375	25,744	1,652	27,886	26.8	4.52	0.534	48.5
B190	Apr. 17-24	64.51	14.060	0.979	Apr. 22	461	20,730	1,897	28,379	26.5	3.06	0.444	45.3
B202	May 3-10	63.80	15.484	1.022	May 7	501	32,700	1,781	27,707	34.0	5.30	0.504	58.1
B214	May 10-16	63.80	15.484	1.048	May 23	457	20,157	1,554	24,062	28.0	4.34	0.400	46.8
B226	June 4-11	70.33	14.508	0.996	June 8	439	30,873	1,626	23,688	20.9	4.36	0.407	40.9
B238	June 20-27	84.48	17.281	1.189	June 25	678	57,274	2,166	37,430	28.6	4.04	0.568	47.8
B250	July 6-13	40.00	10.860	0.698	July 13	400	10,960	1,602	18,375	31.6	3.43	0.381	54.6
B262	July 22-29	77.77	19.028	1.257	July 27	388	30,175	1,665	31,682	28.4	5.40	0.594	47.3
Average.....	.....	.....	.....	.....	.....	.....	31,402	.....	27,938	.....	4.68*	0.530*	51.5*

SUBJECT D.													
B112	Jan. 4-11	165.40	31.241	1.767	Jan. 9	228	37,711	083	30,710	29.3	9.15	0.015	51.8
B123	Jan. 20-27	122.30	25.001	1.458	Jan. 25	262	32,042	092	25,004	45.9	11.80	1.189	.....
B135	Feb. 5-12	147.78	27.703	1.713	Feb. 10	288	42,501	1,308	38,855	44.8	12.45	1.233	.....
B147	Feb. 21-28	153.60	33.379	1.668	Feb. 27	273	41,033	1,152	37,551	25.9	8.65	0.900	53.9
B157	Mar. 8-15	133.30	30.102	1.582	Mar. 11	352	40,043	1,408	42,384	17.5	5.27	0.574	36.3
B169	Mar. 24-31	133.53	20.069	1.484	Mar. 27	322	42,097	1,504	42,089	24.3	6.55	0.786	53.0
B181	Apr. 9-16	128.14	25.236	1.461	Apr. 10	308	39,407	1,532	38,062	21.8	5.50	0.603	47.4
B193	A. 25-M. 2	110.93	26.158	1.420	Apr. 27	252	28,266	1,400	36,021	23.3	6.09	0.677	47.6
B205	May 11-18	120.90	25.113	1.534	May 13	328	39,055	1,624	40,784	24.0	6.03	0.639	41.6
B217	M. 27-J. 3	158.75	29.329	1.534	May 28	339	53,816	1,662	48,745	(34.6)	.....	.....	.....
B229	June 12-19	117.85	28.883	1.503	June 15	265	31,230	1,417	40,027	25.7	7.31	0.818	52.3
B241	J. 28-J. 5	185.48	35.862	1.784	July 1	193	35,797	1,135	40,635	20.7	7.41	0.771	43.2
B253	July 14-21	122.75	27.108	1.398	July 17	307	37,684	1,471	39,876	13.3	3.61	0.411	29.4
B265	July 22-29	157.19	33.715	1.827	July 31	304	47,785	1,448	48,819	16.1	5.43	0.624	34.2
Average.....	.....	.....	.....	.....	.....	.....	39,845	.....	39,518	.....	5.00*	0.677*	43.6*

\* Determinations previous to March 16 are not included in this average.

TABLE 4.—Continued.

SUBJECT E.

LABORATORY NO.	PERIOD	AVERAGE DAILY FECS		AVERAGE DAILY DRY SUBSTANCE		AVERAGE DAILY NITROGEN		DATE BACT. EXAM.	NUMBER OF BACTERIA				GRAVIMETRIC DETERMINATION			
		Gm.	Gm.	Gm.	Gm.	Calculated on Moist Feces			Calculated on Dry Feces		Dry Bacteria in Dry Substance Per cent	Daily Bacteria Gm.	Daily Bacterial Nitrogen Gm.	Bacterial N. in Total Fecal N. Per cent		
						Per Gram. $\times 10^6$	Daily $\times 10^6$		Per Gram. $\times 10^6$	Daily $\times 10^6$						
B118	Jan. 12-10	44.00	13.185	0.797	Jan. 16	454	40,385	....	....	....	....	....	....	....	....	
B130	Jan. 28-F. 4	82.00	23.106	1.378	Jan. 31	404	40,508	1,428	32,005	....	....	....	....	....	....	
B142	Feb. 13-20	99.10	23.325	1.477	Feb. 10	308	30,477	1,723	40,189	46.9	10.84	1.024	69.3	60.3	50.5	
B149	F. 20-M. 7	81.44	23.354	1.442	Mar. 2	480	30,090	1,758	41,050	43.9	10.24	0.728	50.8	50.5	50.5	
B161	Mar. 10-23	110.07	23.319	1.479	Mar. 10	208	32,802	1,574	36,704	20.7	6.94	0.728	50.8	50.5	50.5	
B173	Apr. 1-8	75.62	18.955	1.253	Apr. 1	535	40,459	2,011	38,119	14.5	3.38	0.345	23.3	23.3	23.3	
B185	Apr. 9-16	110.85	23.937	1.621	Apr. 16	441	48,885	1,542	36,911	32.0	7.66	0.866	53.4	53.4	53.4	
B197	A. 25-M. 2	67.11	15.053	1.056	May 5	514	31,412	1,586	23,874	32.0	7.66	0.866	53.4	53.4	53.4	
B209	May 11-18	94.40	23.268	1.547	May 18	513	48,427	1,594	34,905	21.6	5.01	0.506	32.7	32.7	32.7	
B221	May 27-J. 3	78.05	19.117	1.283	June 3	343	36,471	1,478	28,255	31.7	6.06	0.691	53.8	53.8	53.8	
B233	June 12-19	80.06	20.959	1.435	June 19	408	37,469	1,605	34,807	25.6	5.37	0.622	43.4	43.4	43.4	
B245	July 6-13	84.47	17.095	1.202	July 6	373	31,507	1,581	28,450	30.4	5.47	0.624	51.9	51.9	51.9	
B257	July 22-29	86.86	21.016	1.452	July 22	509	40,099	1,976	41,528	26.5	5.57	0.601	41.4	41.4	41.4	
Average.....		.....	.....	.....	.....	.....	37,352	.....	34,824	.....	5.27*	0.580*	42.0*	42.0*	42.0*	

SUBJECT F.

B103	D. 27-J. 3	70.32	19.257	1.366	Dec. 28	459	32,277	.....	.....	.....	.....	.....	.....
B115	Jan. 12-19	66.34	19.730	1.306	Jan. 14	405	30,848	.....	.....	.....	.....	.....	.....
B127	Jan. 28-F. 4	52.94	15.815	1.069	Jan. 29	384	20,338	1,268	19,105	6.01	.....	.....	.....
B139	Feb. 13-20	74.75	20.902	1.482	Feb. 17	409	30,368	1,209	25,271	8.32	0.765	51.6	.....
B153	Feb. 29-Mar. 7	82.80	20.640	1.359	Mar. 6	203	21,770	1,220	25,307	7.10	0.753	55.4	.....
B165	Mar. 10-23	66.79	17.103	1.172	Mar. 23	405	30,455	1,406	25,101	6.47	0.699	59.6	.....
B177	Apr. 1-8	63.42	17.108	1.152	Apr. 6	450	29,403	1,838	31,445	4.82	0.574	49.8	.....
B189	Apr. 17-24	78.14	18.840	1.206	Apr. 22	460	38,913	1,688	31,812	29.2	0.600	46.3	.....
B201	May 3-10	70.12	18.409	1.216	May 7	476	33,386	1,566	28,955	7.40	0.770	63.3	.....
B213	May 19-26	74.97	18.775	1.327	May 23	468	30,500	1,600	30,040	5.28	0.538	40.6	.....
B225	June 4-11	66.50	17.670	1.238	June 8	477	37,240	1,647	30,212	25.2	0.454	36.7	.....
B237	June 20-27	66.45	15.740	1.062	June 25	423	25,570	1,301	21,894	3.68	0.416	30.2	.....



TABLE 4.—Continued.  
SUBJECT 1.

LABORATORY NO.	PERIOD	AVERAGE DAILY FECES	AVERAGE DAILY DRY SUBSTANCE		AVERAGE DAILY NITROGEN	DATE BACT. EXAM.	NUMBER OF BACTERIA				GRAVIMETRIC DETERMINATION			
			Gm.	Gm.			Calculated on Moist Feces		Calculated on Dry Feces		Dry Bacteria in Dry Substance	Daily Dry Bacteria	Daily Bacterial Nitrogen	Bacterial N. in Total Fecal N.
							Per Gram. $\times 10^9$	Daily $\times 10^9$	Per Gram. $\times 10^9$	Daily $\times 10^9$				
B113	Jan. 12-10	67.72	18.338	1.063	Jan. 12	335	22,686	.....	.....	.....	.....	.....	.....	
B125	Jan. 20-27	95.14	22.641	1.310	Jan. 27	200	27,591	1,401	31,720	13.3	3.01	.....	.....	
B137	Feb. 13-20	60.18	22.604	1.439	Feb. 14	408	47,808	1,772	49,161	44.1	9.90	0.019	63.9	
B160	Mar. 8-15	.....	.....	.....	Mar. 14	282	.....	1,476	.....	24.0	.....	.....	.....	
B172	Mar. 24-31	47.11	10.268	0.638	Mar. 31	442	20,823	1,693	17,435	.....	.....	.....	66.8	
B184	Apr. 9-16	77.06	20.881	1.206	Apr. 13	399	31,082	1,541	32,178	35.7	7.45	0.842	66.8	
B196	A. 25-M. 2	58.08	16.424	0.986	Apr. 20	457	26,543	1,405	24,061	24.0	3.94	0.429	43.6	
B208	May 11-18	83.45	14.551	0.884	May 15	478	25,549	1,035	28,150	37.3	5.43	0.554	62.6	
B220	M. 27-J. 3	82.04	21.570	1.324	June 1	397	32,570	1,582	34,124	35.8	7.72	0.531	62.7	
B232	June 12-19	67.07	14.545	0.860	June 17	402	33,296	1,751	25,468	.....	.....	.....	.....	
B244	J. 28-J. 5	104.76	24.129	1.245	July 3	612	64,117	2,250	54,200	28.8	6.95	0.744	50.6	
B256	July 14-21	88.40	18.115	1.240	July 20	576	55,618	2,133	38,639	30.8	5.58	0.642	51.5	
Average.....	.....	.....	.....	.....	.....	.....	36,038	.....	32,646	.....	6.18*	0.674*	57.8*	

SUBJECT J.

B111	Jan. 4-11	136.54	23.725	1.447	Jan. 9	208	40,689	1,006	26,003	28.5	6.76	0.676	46.7
B124	Jan. 20-27	69.51	18.169	1.008	Jan. 25	300	21,479	1,128	20,495	48.1	8.74	0.874	79.6
B136	Feb. 5-12	137.98	23.817	1.503	Feb. 10	220	30,356	1,302	33,153	44.6	10.62	1.062	68.0
B148	Feb. 21-28	98.90	20.015	1.356	Feb. 27	386	38,175	1,317	27,545	45.5	9.52	0.952	70.2
B159	Mar. 8-15	89.83	19.049	1.107	Mar. 13	364	32,608	1,201	24,502	28.1	5.35	0.535	45.9
B171	Mar. 24-31	62.86	15.917	0.907	Mar. 30	401	25,297	1,373	21,854	34.4	5.48	0.559	57.8
B183	Apr. 9-16	66.20	15.604	0.900	Apr. 14	439	29,062	1,287	20,108	26.8	4.21	0.450	45.5
B185	Apr. 25-M. 2	82.63	18.943	1.213	Apr. 20	302	27,974	1,302	24,664	35.0	6.63	0.709	58.5
B207	May 11-18	88.38	18.084	1.206	May 15	383	33,850	1,710	30,024	38.8	7.02	0.730	60.5
B210	May 27-J. 3	70.64	17.423	1.079	June 1	476	33,625	1,817	31,658	34.5	6.01	0.619	57.4
B231	June 12-19	76.14	17.840	1.118	June 17	368	28,020	1,484	26,475	.....	.....	.....	.....
B243	June 28-J. 5	102.04	17.768	1.222	July 4	372	37,959	1,738	30,881	23.4	4.16	0.437	35.7
B255	July 14-21	76.74	16.158	1.068	July 20	228	17,497	1,416	22,880	19.2	3.10	0.335	31.3
Average.....	.....	.....	.....	.....	.....	.....	30,597	.....	26,256	.....	5.23*	0.548*	49.5*



[illegible][illegible]

\* Determinations previous to March 16 are not included in this average.

in Subject A,  $28 \times 10^{12}$ , and in Subject B,  $29 \times 10^{12}$ . The average number of daily fecal bacteria for the 12 individuals is  $35 \times 10^{12}$ .

In those instances where the average daily number of bacteria was also calculated upon the basis of fecal dry substance, the values are in general slightly lower than those calculated upon the basis of moist feces, and the range of variation is not quite so wide. The highest values for daily fecal bacteria, calculated on the basis of dry substance, occur in Subject H, January 6,  $58 \times 10^{12}$  bacteria per day, and Subject I, July 3,  $54 \times 10^{12}$ . A value of  $51 \times 10^{12}$  occurs in Subject H on June 12. The lowest values occur in Subject B, April 16,  $14 \times 10^{12}$  bacteria per day, and in Subject I, March 31,  $17 \times 10^{12}$  bacteria per day. Of the 12 individuals, Subject H still shows the highest average value,  $40 \times 10^{12}$ ; Subject B shows the lowest,  $26 \times 10^{12}$  bacteria per day. The grand average of the 12 subjects for all examinations in which the number of daily bacteria was calculated on the basis of dry substance, is  $32 \times 10^{12}$  bacteria per day, about 9 per cent lower than when calculated on the basis of moist feces.

The daily bacterial dry substance of the feces has been calculated from the Strasburger determinations on the basis of dry substance throughout. The highest daily bacterial dry substance is shown by Subject H, June 12, 9.15 gm., containing 1.006 gm. of nitrogen. On April 25, the same individual excreted 8.65 gm. of dry bacterial substance, containing 0.960 gm. of nitrogen. The lowest value is in Subject B, on April 16, 1.87 gm. of dry bacterial substance containing 0.194 gm. of nitrogen. This same subject excreted on May 18, 2.66 gm., and on June 3, 2.81 gm. of dry bacterial substance containing 0.303 and 0.334 gm. of nitrogen respectively. For the whole period of observation, Subject H shows the highest average daily bacterial dry substance, 7.26 gm. containing 0.819 gm. of nitrogen; Subject B shows the lowest average, 3.56 gm. dry bacterial substance containing 0.393 gm. of nitrogen. The average of the 12 subjects is 5.34 gm. daily bacterial dry substance, representing 0.585 gm. of nitrogen.

The portion of the total fecal nitrogen contained in the fecal bacteria varied in the single examinations from 66.8 per cent in Subject I, April 13, to 23.3 per cent in Subject K, June 5. Subject I shows the highest average value for all examinations, 57.4 per cent, Subject K the lowest, 32.1 per cent. The grand average for the 12 individuals

is 46.3 per cent of the total fecal nitrogen, contained in the fecal bacteria.

The following table, 5, summarizes briefly the extreme variations and the mean of these observations.

TABLE 5.  
SUMMARY OF DAILY FECAL BACTERIA.

	NUMBER CALCULATED ON		DRY BACTERIAL SUBSTANCE  Grams	BACTERIAL NITROGEN	
	Moist Feces	Dry Feces		Daily Quantity  Grams	In Total Fecal Nitrogen Per cent
Mean.....	$35 \times 10^{12}$	$32 \times 10^{12}$	5.34	0.585	46.3
Maximum.....	$64 \times 10^{12}$	$58 \times 10^{12}$	9.15	1.006	66.8
Minimum.....	$16 \times 10^{12}$	$14 \times 10^{12}$	1.87	0.194	23.3

#### DIFFERENTIAL COUNTS.

Strasburger<sup>20</sup> has studied Gram stained microscopic preparations of adult feces. He found the bacteria predominantly Gram negative, especially when the diet consisted largely of milk and vegetables. In the case of a meat diet he found an increased number of Gram positive micrococci.

Table 6, A to L, shows the results of differential counts of Gram stained preparations of mixed fecal flora from 157 different stools. The classes of bacteria, designated in the table, were in general quite easily distinguished from each other except where the distinction rested solely upon the color. In these cases there was some difficulty to which attention has been directed in describing the method of staining.

Bacilli 0.4 to 0.9  $\mu$  wide and from 1.0 to 2.5  $\mu$  long were classed as *B. coli* type. The quantity of these varied from 81.6 per cent in Subject L, January 20, 1908, to 19 per cent in Subject E, April 1, 1908. In the second class, all the remaining Gram negative rods were placed. The quantity of these varied from 46.2 per cent in Subject E on March 2, 1908, to 0.4 per cent in Subject H on January 6, 1908. The distinction between these two classes is not sharp. The Gram negative micrococci varied from 36.8 per cent in Subject H on January 6, to 0.8 per cent in Subject L on June 12, 1908. The distinction between the bacteria of this class and the bacteria classified as Gram positive cocci was not accurate and frequently exceedingly difficult. Spirilla

TABLE 6.  
DIFFERENTIAL COUNT OF FECAL BACTERIA.  
SUBJECT A.

Laboratory No.	B110	B122	B134	B146	B158	B170	B182	B104	B206	B218	B230	B242	B254	B266	Average
Date	Jan. 6	Jan. 22	Feb. 6	Feb. 24	Mar. 11	Mar. 27	Apr. 10	Apr. 27	May 13	May 28	June 15	July 1	July 17	July 31	Per cent
<i>Bacillus coli</i> type...	47.8	54.0	62.6	36.0	42.6	38.7	39.8	32.8	45.4	33.4	36.6	43.8	37.2	42.4	42.4
Other negative rods...	12.3	14.6	17.6	36.2	22.6	36.5	32.8	37.8	26.8	31.6	32.8	24.4	27.8	27.2	27.2
Negative cocci...	12.8	7.5	9.0	4.4	5.2	1.4	1.6	5.8	2.4	4.4	7.8	6.0	3.8	5.6	5.6
Negative spirochetes...	9.0	0.0	0.4	0.4	0.8	0.4	0.0	0.0	0.6	0.0	0.0	0.0	0.0	0.4	0.4
Gram positive rods...	18.8	11.9	3.8	5.6	6.2*	5.0	8.8*	8.0	4.2	4.6	3.8	6.8	3.8	6.2	6.2
Positive cocci...	18.8	9.2	0.4	3.8	15.4	14.2	10.4	10.8	10.2	10.8	15.6	16.4	14.4	12.4	12.4
Oval free spores...	0.0	1.2	4.8	5.0	3.4	3.5	4.8	3.8	8.2	8.6	3.2	2.4	4.0	4.0	4.0
Spherical free spores...	0.2	1.6	1.4	8.6	3.8	0.3	1.8	1.0	2.2	0.6	0.2	0.2	0.8	1.7	1.7
Total Gram negative...	72.9	76.1	89.6	77.0	71.2	77.0	74.2	70.4	75.2	69.4	77.2	74.2	72.0	75.0	75.0
Total Gram positive...	26.9	21.1	4.2	0.4	21.6	19.2	19.2	18.8	14.4	21.4	10.4	23.2	23.2	18.8	18.8
Total free spores...	0.2	2.8	6.2	13.6	7.2	3.8	6.6	4.8	10.4	9.2	3.4	2.6	4.8	5.8	5.8
Total negative rods...	60.1	68.6	80.2	72.2	65.2	75.2	72.6	70.6	72.2	65.0	69.4	68.2	65.0	60.0	60.0
Total micrococci...	31.6	16.7	9.4	8.2	20.6	15.6	12.0	10.6	12.6	21.2	23.4	32.4	25.2	18.0	18.0
Quality of the stain...	...	...	...	Poor	...	Fair	Poor	Exc.	Good	Good	Good	Good	Fair	...	...

SUBJECT B.

Laboratory No.	B114	B126	B138	B150	B162	B174	B186	B108	B210	B222	B234	B246	B258	Average
Date	Jan. 12	Jan. 27	Feb. 13	Mar. 2	Mar. 16	Apr. 1	Apr. 16	May 1	May 18	June 3	June 10	July 6	July 22	Per cent
<i>Bacillus coli</i> type...	44.0	30.4	37.2	31.6	42.2	40.4	41.2	30.6	44.4	39.4	31.6	35.4	41.6	39.1
Other negative rods...	29.0	21.4	17.6	31.8	26.4	25.8	24.8	32.6	34.4	28.0	34.6	36.6	30.2	28.7
Negative cocci...	18.4	26.2	19.2	11.0	21.8	9.8	6.6	5.8	4.8	3.6	2.2	5.6	4.8	10.7
Negative spirochetes...	0.0	3.8	4.0	2.2	0.6	0.2	0.4	0.0	0.4	0.4	0.0	0.0	0.6	0.8
Gram positive rods...	4.8	5.4*	4.0	5.0	3.4	2.4	3.4	4.4	6.0	7.2	4.0	6.8	5.2	4.8
Positive cocci...	3.2	6.0	16.8	17.8	4.0	20.8	22.6	17.0	9.4	20.8	26.8	15.0	17.0	15.2
Oval free spores...	0.6	0.2	0.6	0.2	0.4	0.0	0.8	0.0	0.6	0.4	0.4	0.4	0.6	0.4
Spherical free spores...	0.0	0.0	0.2	0.6	1.2	0.6	0.2	0.6	0.0	0.2	0.4	0.2	0.0	0.4
Total Gram negative...	91.4	88.4	77.8	76.6	91.0	76.2	73.0	78.0	84.0	71.4	68.4	77.6	77.2	79.3
Total Gram positive...	8.0	11.4	21.4	22.8	7.4	23.2	26.0	21.4	15.4	28.0	30.8	21.8	22.2	20.0
Total free spores...	0.6	0.2	0.8	0.6	1.6	0.6	1.0	0.6	0.6	0.6	0.8	0.6	0.6	0.7
Total negative rods...	73.0	60.8	54.8	63.4	68.6	66.2	66.0	72.2	78.8	67.4	66.2	72.0	71.8	67.8
Total micrococci...	21.6	32.2	36.0	28.8	25.8	30.6	29.2	22.8	14.2	24.4	29.0	20.6	21.8	25.0
Quality of the stain...	...	...	...	Good	...	Good	Fair	Poor	Good	Good	Good	Good	Good	...

SUBJECT C.

Laboratory No. ....	B104	B116	B128	B140	B154	B166	B178	B100	B202	B214	B226	B238	B250	B262	Average
Date .....	Dec. 28	Jan. 14	Jan. 29	Feb. 20	Mar. 6	Mar. 23	Apr. 6	Apr. 22	May 7	May 23	June 8	June 25	July 13	July 27	Per cent
<i>Bacillus coli</i> type .....	62.6	67.0	55.2	51.2	53.6	52.0	35.6	54.0	33.4	39.4	39.8	55.4	39.0	36.8	48.2
Other negative rods .....	29.0	8.2	11.8	27.0	26.4	35.0	30.2	31.2	33.3	26.0	32.0	16.0	35.2	25.6	25.6
Negative cocci .....	7.2	10.8	2.0	7.5	4.8	1.8	4.6	5.6	7.7	6.0	5.4	3.2	4.2	1.2	5.2
Negative spirochetes .....	1.2	2.4	0.2	0.2	0.2	4.0	5.4	1.2	1.4	0.4	1.0	1.0	0.4	1.4	1.8
Gram positive rods .....	2.6	3.4	11.4	5.0	2.2	2.6	5.0	2.4	4.7	6.8	5.0	5.2	3.4	2.4	4.4
Positive cocci .....	4.6	2.6	18.8	8.8	6.8	3.2	17.6	2.8	18.0	19.6	15.2	17.0	10.8	31.6	13.1
Oval free spores .....	1.0	3.4	0.4	0.3	1.0	0.8	1.0	2.8	1.2	1.4	1.4	1.6	1.0	0.4	1.3
Spherical free spores .....	0.8	2.2	0.2	0.2	0.0	0.0	0.6	0.0	0.3	0.2	0.2	0.6	0.0	0.6	0.4
Total Gram negative .....	01.0	88.4	60.2	85.7	90.0	93.4	75.8	02.0	75.8	72.0†	78.2	75.6	78.8	65.0	80.8
Total Gram positive .....	7.2	6.0	30.2	13.8	9.0	5.8	22.6	5.2	22.7	26.4	22.2	26.4	20.2	34.0	17.5
Total free spores .....	1.8	5.6	0.6	0.5	1.0	0.8	1.6	2.8	1.5	1.6	1.6	2.2	1.0	1.0	1.7
Total negative rods .....	82.6	75.2	67.0	78.2	80.0	87.0	65.8	85.2	66.7	65.4	71.8	71.4	74.2	62.4	73.8
Total micrococci .....	11.8	13.4	20.8	16.3	11.6	5.0	23.2	8.4	25.4	25.6	20.6	20.2	21.0	32.8	18.3
Quality of the stain .....	.....	.....	.....	.....	Poor	Good	Fair	Poor	Good	Good	Exc.	Fair	Good	Fair	.....

SUBJECT D.

Laboratory No. ....	B112	B123	B135	B147	B157	B169	B181	B193	B205	B217	B229	B241	B253	B265	Average
Date .....	Jan. 9	Jan. 25	Feb. 10	Feb. 27	Mar. 11	Mar. 27	Apr. 10	Apr. 27	May 13	May 28	June 15	July 1	July 17	July 31	Per cent
<i>Bacillus coli</i> type .....	67.6	49.2	48.4	40.0	45.8	41.0	42.8	53.8	46.4	47.4	34.6	50.6	42.2	28.4	45.8
Other negative rods .....	2.0	16.0	18.0	27.8	20.6	27.0	20.4	20.8	20.6	20.6	34.4	23.6	35.2	40.0	24.0
Negative cocci .....	12.8	16.0	22.4	14.6	17.4	10.8	8.8	6.6	6.8	3.6	3.0	4.2	5.0	6.2	9.0
Negative spirochetes .....	0.0	0.4	1.6	1.4	1.0	2.0	1.6	2.0	7.6	0.0	3.6	5.8	0.0	7.2	2.4
Gram positive rods .....	8.2	7.8	4.4	11.0	6.4	4.0	8.4	8.0	0.0	8.0	0.2	7.6	7.6	3.6	7.4
Positive cocci .....	8.6	9.4	3.4	5.0	6.6	11.0	18.0	8.4	0.2	10.6	13.4	8.0	0.2	13.6	9.6
Oval free spores .....	0.6	0.4	0.2	0.2	0.2	0.0	0.0	0.4	0.4	0.6	0.6	0.0	0.4	0.8	0.4
Spherical free spores .....	0.2	0.8	1.4	0.0	2.0	1.2	0.0	0.0	0.0	0.2	1.2	0.2	0.4	0.2	0.5
Total Gram negative .....	82.4	81.6	90.4	83.8	84.8	83.8	73.6	83.2	81.4	80.6	75.6	84.2	82.4	81.8	82.1
Total Gram positive .....	16.8	17.2	7.8	16.0	13.0	15.0	26.4	16.4	18.4	18.6	22.6	15.6	16.8	17.2	17.0
Total free spores .....	0.8	1.2	1.8	0.2	2.2	1.2	0.0	0.4	0.4	0.8	1.8	0.2	0.8	1.0	0.9
Total negative rods .....	60.6	65.2	66.4	67.8	66.4	71.0	63.2	74.6	67.0	77.0	60.0	74.2	77.4	68.4	60.8
Total micrococci .....	21.4	25.4	25.8	19.6	24.0	21.8	26.8	15.0	16.0	14.2	16.4	12.2	14.2	10.8	10.5
Quality of the stain .....	.....	.....	.....	Good	Good	Good	Good	Good	Good	Good	Good	Fair	Fair	Good	.....

\* One of these bacilli (0.2 per cent. of the bacteria counted) contained a spore.

† A single Gram negative spirillum was observed in counting 500 bacteria in this stool. This 0.2 per cent. is included in the total Gram negative bacteria.

TABLE 6 (Continued).  
SUBJECT E.

Laboratory No. ....	B106	B118	B130	B142	B149	B161	B173	B185	B197	B209	B221	B233	B245	B257	Average
Date .....	Dec. 30	Jan. 16	Jan. 31	Feb. 10	Mar. 2	Mar. 16	Apr. 1	Apr. 16	May 1	May 18	June 3	June 10	July 6	July 22	Per cent
<i>Bacillus coli</i> type.....	54.0	63.2	Count	42.8	20.6	42.8	10.0	48.4	53.0	Count	30.2	30.8	60.2	31.4	43.6
Other negative rods.....	16.6	8.2	not made	38.4	46.2	31.6	45.0	28.6	31.4	not made	30.2	33.8	27.8	38.2	31.3
Negative cocci.....	10.0	13.2	Count	12.2	0.2	5.6	0.0	8.8	6.6	Count	6.4	3.8	5.2	5.4	7.6
Negative spirochetes.....	2.0	0.8	Count	0.2	0.2	0.0	0.0	0.2	0.6	Count	0.0	0.0	0.6	0.8	0.8
Gram positive rods.....	6.6	2.4	Count	1.6	2.8*	0.0	4.2	3.8	2.4	Count	7.8	3.0	2.0	3.8	3.8
Positive cocci.....	9.6	7.2	Count	1.2	12.0	13.0	24.2	7.8	4.6	Count	14.2	18.2	3.0	18.4	11.2
Oval free spores.....	0.6	2.2	Count	2.0	0.6	0.6	1.0	2.2	0.6	Count	1.8	1.2	0.4	2.0	1.3
Spherical free spores.....	0.6	2.8	Count	1.6	0.2	0.4	0.0	0.2	0.8	Count	0.4	0.2	0.8	0.0	0.7
Total Gram negative.....	82.6	85.4	Count	93.6	84.4	80.0	70.0	86.0	91.6	Count	75.8	77.4	93.8	75.8	83.0
Total Gram positive.....	16.2	9.6	Count	2.8	14.8	10.0	28.4	11.6	7.0	Count	22.0	21.2	5.0	22.2	15.0
Total free spores.....	1.2	5.0	Count	3.6	0.8	1.4	64.0	2.4	1.4	Count	2.2	1.4	1.2	2.0	2.0
Total negative rods.....	70.6	71.4	Count	81.2	75.8	74.4	64.0	77.0	84.4	Count	69.4	73.6	88.0	69.6	74.9
Total micrococci.....	19.6	20.4	Count	13.4	20.4	18.6	30.2	10.6	11.2	Count	20.6	22.0	8.2	23.8	18.8
Quality of the stain.....	.....	.....	.....	Good	Good	.....	Good	Poor	Poor	.....	Good	Good	Fair	Good	.....

SUBJECT F.

Laboratory No. ....	B103	B115	B127	B139	B153	B165	B177	B189	B201	B213	B225	B237	B249	B261	Average
Date .....	Dec. 28	Jan. 14	Jan. 29	Feb. 17	Mar. 6	Mar. 23	Apr. 6	Apr. 22	May 7	May 23	June 8	June 25	July 13	July 27	Per cent
<i>Bacillus coli</i> type.....	53.4	44.8	41.4	.....	40.6	41.6	52.4	42.4	42.8	30.4	45.2	37.8	40.0	44.4	41.0
Other negative rods.....	22.6	23.0	14.8	.....	14.6	20.2	18.0	25.8	20.0	30.6	21.8	27.8	32.4	25.4	23.0
Negative cocci.....	10.4	14.8	20.6	.....	21.4	17.2	10.0	10.8	5.2	3.8	7.2	6.4	3.8	7.4	10.7
Negative spirochetes.....	1.0	0.0	1.0	.....	0.4	8.8	0.0	0.6	0.6	0.0	0.0	0.0	0.0	0.0	0.3
Gram positive rods.....	2.2	7.8	10.4	.....	11.0	8.0	6.2	10.0	12.0	9.2	8.0	6.2	6.4	11.6	8.1
Positive cocci.....	8.0	7.4	9.6	Count	10.6	10.2	11.2	8.8	17.0	15.8	17.0	19.2	11.0	10.6	12.0
Oval free spores.....	1.6	1.6	0.8	Count	0.6	0.6	1.8	0.4	1.0	0.4	0.8	1.4	0.4	0.2	0.9
Spherical free spores.....	0.8	0.6	1.4	Count	1.0	1.4	0.4	1.2	1.4	0.8	0.4	1.2	0.0	0.4	0.8
Total Gram negative.....	87.4	82.6	77.8	Count	77.0	79.8	80.4	70.6	68.6	73.8	74.2	72.0	82.2	77.2	77.0
Total Gram positive.....	10.2	15.2	20.0	Count	21.4	18.2	17.4	18.8	20.0	25.0	25.0	25.4	17.4	22.2	20.4
Total free spores.....	2.4	2.2	2.2	Count	1.4	2.0	2.2	1.6	2.4	1.2	0.8	2.6	0.4	0.6	1.7
Total negative rods.....	76.0	67.8	56.2	Count	55.2	61.8	70.4	68.2	62.8	70.0	67.0	65.6	78.4	69.8	66.0
Total micrococci.....	18.4	22.6	30.2	Count	35.0	27.4	21.2	10.6	22.2	10.6	24.2	25.6	14.8	18.0	22.7
Quality of the stain.....	.....	.....	.....	.....	.....	Fair	Poor	Good	Good	Good	Good	Good	Fair	Good	.....



SUBJECT G.

Laboratory No.	B119	B131	B143	B152	B164	B176	B188	B200	B212	B224	B236	B248	B264	Average
Date	Jan. 20	Feb. 3	Feb. 21	Mar. 4	Mar. 19	Apr. 3	Apr. 20	May 4	May 20	June 5	June 22	July 9	July 29	
	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
<i>Bacillus coli</i> type	46.0	47.6	51.8	30.0	32.4	34.6	39.4	63.2	52.4	52.0	42.0	41.2	54.2	45.1
Other negative rods	16.0	22.4	17.6	20.2	30.8	32.0	10.8	22.0	20.8	25.2	28.8	32.3	27.8	25.0
Negative cocci	11.8	10.8	5.6	3.2	7.4	8.4	5.2	4.4	9.4	5.6	3.8	11.2	10.4	7.5
Negative spirochetes	3.2	3.4	1.0	1.0	1.0	0.6	1.6	0.2	0.2	0.2	0.6	0.3	0.6	1.1
Gram positive rods	6.4†	4.4†	5.8	11.6†	7.6	4.4	5.8†	2.0	4.8	5.0	5.0	5.2	1.0	5.3§
Positive cocci	10.6	4.4	13.0	21.6	16.8	15.6	26.0	6.2	4.8	8.6	17.4	8.1	4.0	12.4
Oval free spores	1.6	4.4	2.6	1.4	0.8	4.4	2.2	1.0	2.8	3.0	0.4	1.5	1.6	2.4
Spherical free spores	4.4	2.6	2.6	2.0	0.8	0.0	0.0	1.0	0.2	0.4	0.2	0.2	0.4	1.2
Total Gram negative	77.0	84.2	76.0	63.4	72.2	75.6	66.0	89.8	82.8	83.0	75.2	85.0	93.0	78.7
Total Gram positive	17.0	8.8	18.8	33.2	24.4	20.0	31.8	8.2	14.2	13.6	22.4	13.3	5.0	17.7
Total free spores	6.0	7.0	5.2	3.4	3.4	4.4	2.2	2.0	3.0	3.4	2.4	1.7	2.0	3.6
Total negative rods	62.0	70.0	60.4	59.2	63.2	66.6	59.2	85.2	73.2	77.2	70.8	73.5	82.0	70.1
Total micrococci	22.4	15.2	18.6	24.8	24.2	24.0	31.2	10.6	18.8	14.2	21.2	19.3	14.4	19.9
Quality of the stain	....	Fair	....	Good	Good	Fair	Good	Fair	Good	Fair	Exc.	Good	Poor	

SUBJECT H.

Laboratory No.	B109	B121	B133	B145	B156	B168	B180	B192	B204	B216	B228	B240	B252	B260	Average
Date	Jan. 6	Jan. 22	Feb. 6	Feb. 24	Mar. 10	Mar. 25	Apr. 8	Apr. 25	May 11	May 25	June 12	June 20	July 15	July 24	
	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
<i>Bacillus coli</i> type	58.4	44.6	48.4	43.8	43.4	51.6	Count not made	48.2	45.6	47.2	32.8	48.4	50.0	48.8	47.0
Other negative rods	0.4	12.2	16.4	18.6	35.4	33.2	4.8	28.0	20.6	31.2	34.2	23.4	24.2	27.6	24.2
Negative cocci	36.8	11.2	22.8	18.8	3.6	4.8	7.0	7.0	3.6	4.8	12.0	4.4	4.4	12.2	11.2
Negative spirochetes	0.0	1.8	0.4	1.2	1.8	0.4	0.6	0.6	0.8	0.2	0.0	0.0	0.0	0.4	0.6
Gram positive rods	0.0	13.0	6.2	7.0	4.2	2.8	3.2	3.2	5.2	5.6	5.6	4.0	3.8	1.8	4.8
Positive cocci	2.6	15.8	5.6	9.4	0.2	5.6	Count not made	11.4	13.0	7.8	14.0	18.4	16.0	7.8	10.5
Oval free spores	1.4	0.0	0.0	0.4	2.0	1.4	1.2	1.2	2.0	3.0	1.4	1.4	1.4	1.4	1.4
Spherical free spores	0.4	0.6	0.2	0.8	0.4	0.2	0.4	0.4	0.2	0.2	0.0	0.4	0.2	0.0	0.3
Total Gram negative	95.6	69.8	88.0	82.4	84.2	90.0	83.8	79.6	78.6	83.4	79.0	76.2	78.6	89.0	83.0
Total Gram positive	2.6	28.8	11.8	16.4	13.4	8.4	14.6	14.6	19.2	13.4	10.6	22.4	19.8	9.6	15.3
Total free spores	1.8	1.4	0.2	1.2	2.4	1.6	1.6	1.6	2.2	3.2	1.4	1.4	1.6	1.4	1.7
Total negative rods	58.8	56.8	64.8	62.4	78.8	84.8	76.2	75.2	78.4	78.4	67.0	71.8	74.2	76.4	70.1
Total micrococci	39.4	27.0	28.4	38.2	12.8	10.4	18.4	16.6	16.6	12.6	26.0	22.8	20.4	20.0	21.7
Quality of the stain	....	....	Good	Fair	....	Good	Fair	Fair	Good	Fair	Good	Good	Fair	Fair	

\* Four of these bacilli (0.8 per cent. of the bacteria counted) contained spores.

† Two of these bacilli (0.4 per cent. of the bacteria counted) contained spores.

‡ Three of these bacilli (0.6 per cent. of the bacteria counted) contained spores.

§ One of these bacilli (0.2 per cent. of the bacteria counted) contained a spore.

TABLE 6. (Continued).  
SUBJECT I

Laboratory No.	B101	B113	B125	B137	B160	B172	B184	B106	B208	B220	B232	B244	B256	Average
Date	Dec. 20	Jan. 12	Jan. 27	Feb. 14	Mar. 14	Mar. 31	Apr. 13	Apr. 29	May 15	June 1	June 17	July 3	July 20	Per cent
<i>Bacillus coli</i> type.....	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
Other negative rods.....	63.0	69.0	69.8	48.4	50.4	46.2	46.2	38.8	43.0	42.1	45.0	34.0	43.0	49.2
Negative cocci.....	14.6	3.6	16.4	35.8	14.8	10.4	24.6	34.6	27.0	30.8	30.0	44.4	34.2	25.4
Negative spirochetes.....	10.2	0.8	2.6	2.4	19.0	9.4	7.2	0.0	6.0	5.2	1.4	6.0	4.4	6.0
Gram positive rods.....	0.2	1.4	0.0	0.8	0.6	0.2	0.4	0.2	0.8	0.0	0.0	0.0	0.0	0.4
Positive cocci.....	2.9	1.0	2.8	3.2	7.6	4.8	6.2	8.0	4.8	0.0	4.0	6.2	7.0*	5.0
Oval free spores.....	3.1	3.4	3.8	1.0	5.2	10.0	14.0	9.0	10.8	14.2	17.2	7.2	10.4	9.3
Spherical free spores.....	4.2	3.2	3.8	7.0	1.2	0.6	1.0	2.8	1.6	1.2	1.8	2.2	0.6	2.4
Total Gram negative.....	1.8	10.0	1.8	1.4	1.2	0.4	0.4	0.6	0.0	0.0	0.6	0.0	0.4	1.4
Total Gram positive.....	88.0	83.8	88.8	87.4	84.8	75.2	78.4	79.6	70.8	78.4	76.4	84.4	81.6	81.0
Total free spores.....	6.0	3.0	5.6	4.2	12.8	23.8	20.2	17.0	21.6	20.2	21.2	13.4	17.4	14.3
Total negative rods.....	6.0	13.2	5.6	8.4	2.4	1.0	1.4	3.4	1.6	1.4	2.4	2.2	1.0	3.8
Total micrococci.....	77.6	72.6	80.2	84.2	65.2	65.6	70.8	73.4	70.0	73.2	75.0	78.4	77.2	74.6
Quality of the stain.....	13.3	11.2	5.4	3.4	24.2	28.4	21.2	15.0	22.8	9.4	18.0	13.2	14.8	16.2

SUBJECT J.

Laboratory No.	B100	B111	B124	B136	B148	B150	B171	B183	B195	B207	B210	B231	B243	B255	Average
Date	Dec. 23	Jan. 9	Jan. 25	Feb. 10	Feb. 27	Mar. 13	Mar. 30	Apr. 14	Apr. 29	May 15	June 1	June 17	July 4	July 20	Per cent
<i>Bacillus coli</i> type.....	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
Other negative rods.....	51.8	68.2	Count not made	45.8	52.4	40.8	47.0	31.8	44.0	47.8	50.0	40.5	32.6	33.8	45.8
Negative cocci.....	31.2	3.2	Count not made	21.2	38.6	40.0	31.0	31.6	31.4	27.4	22.2	22.2	35.6	21.2	27.6
Negative spirochetes.....	11.0	6.8	Count not made	12.8	6.0	4.4	4.4	3.0	4.4	9.2	7.2	5.2	1.2	10.8	6.6
Gram positive rods.....	2.4	0.8	Count not made	10.6	0.4	1.0	0.4	3.4	0.6	4.0	0.4	0.5	4.2	11.2	2.8
Positive cocci.....	5.6	8.6	Count not made	3.8	1.4	1.6	2.8	3.2	6.2	4.6	2.6	4.8	7.2	6.2	4.6
Oval free spores.....	1.2	0.8	Count not made	4.2	0.0	7.6	8.6	18.2	11.0	8.0	5.8	18.2	18.2	15.6	10.0
Spherical free spores.....	0.6	3.0	Count not made	1.2	1.2	3.4	4.8	2.4	1.0	0.6	2.0	0.4	0.4	1.2	1.7
Total Gram negative.....	86.6	79.0	Count not made	90.4	97.4	80.2	83.6	72.8	80.4	84.6	88.8	76.2	73.6	77.0	82.8
Total Gram positive.....	11.8	17.2	Count not made	8.0	1.4	9.2	11.4	22.4	17.2	13.2	23.0	25.4	21.8	14.6	14.6
Total free spores.....	1.8	3.8	Count not made	1.6	1.2	4.6	5.0	4.8	2.4	2.2	2.8	0.8	1.0	1.2	2.6
Total negative rods.....	73.0	71.4	Count not made	67.0	91.0	86.8	78.8	66.4	75.4	75.2	81.2	70.5	68.2	55.0	73.4
Total micrococci.....	17.2	15.4	Count not made	17.0	6.0	12.0	13.0	21.2	13.4	17.8	13.0	23.4	10.4	26.4	16.7
Quality of the stain.....	....	....	Count not made	....	....	Poor	Poor	Poor	Fair	Fair	Poor	Fair	Good	Good	....

SUBJECT K.

Laboratory No.	B177	B120	B141	B151	B103	B175	B187	B109	B211	B223	B235	B247	B203	Average
Date	Jan. 16	Jan. 31	Feb. 19	Mar. 4	Mar. 19	Apr. 3	Apr. 20	May 4	May 20	June 5	June 23	July 9	July 29	Per cent
<i>Bacillus coli</i> type.....	51.0	37.4	40.2	40.2	44.7	51.6	58.0	50.2		44.0	42.4	55.2	40.8	47.1
Other negative rods.....	20.0	10.8	10.2	20.6	19.3	18.4	17.4	24.4		26.6	32.4	22.8	25.4	22.5
Negative cocci.....	16.4	17.2	10.2	15.6	17.0	12.4	5.4	6.4		4.4	3.6	3.4	5.6	10.5
Negative spirochetes.....	0.2	0.2	1.4	1.2	1.2	0.8	0.4	0.2		0.0	0.0	0.4	0.4	1.3
Gram positive rods.....	4.8	11.2	3.0	8.6	5.1	5.6	7.2	8.8		11.8	7.4	6.0	4.8	7.0
Positive cocci.....	7.0	12.2	9.2	12.8	12.0	10.4	10.2	8.2		10.8	12.8	10.8	2.8	9.9
Oval free spores.....	0.4	0.2	0.6	0.2	0.8	0.4	0.8	0.4		2.2	1.0	0.6	1.0	0.7
Spherical free spores.....	0.2	1.8	7.2	0.8	0.7	0.4	0.6	1.4		0.2	0.4	0.8	0.6	1.3
Total Gram negative.....	87.6	74.6	80.0	77.6	82.2	83.2	81.2	81.2		75.0	78.4	81.8	90.2	81.1
Total Gram positive.....	11.8	23.4	12.2	21.4	17.1	16.0	17.4	17.0		22.6	20.2	16.8	7.6	16.9
Total free spores.....	0.6	2.0	7.8	1.0	0.7	0.8	1.4	1.8		2.4	1.4	1.4	2.2	2.0
Total negative rods.....	71.0	57.2	59.4	60.8	64.0	70.0	75.4	74.6		70.6	74.8	78.0	75.2	69.3
Total micrococci.....	23.4	20.4	28.4	28.4	29.0	22.8	15.6	14.6		15.2	16.4	14.2	8.4	20.5
Quality of the stain.....	.....	.....	.....	Fair	Fair	.....	Good	Good		Fair	Good	Good	Good	.....

SUBJECT L.

Laboratory No.	B108	B120	B132	B144	B155	B167	B179	B101	B203	B215	B227	B239	B251	B259	Average
Date	Jan. 2	Jan. 20	Feb. 3	Feb. 21	Mar. 9	Mar. 25	Apr. 8	Apr. 24	May 11	May 25	June 12	June 20	July 15	July 25	Per cent
<i>Bacillus coli</i> type.....	46.2	81.6	50.2	46.8	40.6	53.4	52.8	58.0	54.8	58.6	48.6	57.6	46.2	45.0	52.9
Other negative rods.....	20.2	7.0	1.0	18.2	10.2	18.8	15.2	21.0	20.4	14.4	27.2	22.0	20.0	33.8	19.5
Negative cocci.....	20.2	3.6	20.6	11.8	11.6	18.6	13.6	8.0	3.4	3.8	0.8	3.6	7.0	4.2	9.3
Negative spirochetes.....	1.0	0.6	1.6	1.8	1.4	0.2	0.4	0.0	0.2	0.6	0.2	0.0	0.2	0.0	0.5
Gram positive rods.....	8.8	3.0	6.8	0.8	0.8	0.4	8.0	6.2	8.4	0.6	8.2	6.2	4.8	6.8	7.1
Positive cocci.....	2.6	2.2	8.6	0.0	10.0	3.2	9.4	5.0	11.0	14.2	13.0	9.8	14.6	9.0	9.2
Oval free spores.....	0.4	1.0	0.2	0.6	0.2	1.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.6
Spherical free spores.....	0.6	0.8	1.2	2.0	0.2	1.4	0.0	0.0	1.2	0.8	0.8	0.2	0.6	0.2	0.9
Total Gram negative.....	87.6	92.8	83.4	78.6	72.8	88.0	82.0	87.6	78.8	77.4	70.8	83.2	79.4	83.0	82.2
Total Gram positive.....	11.4	5.4	15.4	18.8	25.8	9.6	17.4	11.2	19.4	20.8	21.8	16.0	19.4	15.8	16.3
Total free spores.....	1.6	1.8	1.2	2.0	1.4	2.4	0.6	1.2	1.8	1.8	1.4	0.6	1.2	1.2	1.5
Total negative rods.....	66.4	89.0	61.2	65.0	59.8	69.3	68.0	79.6	75.2	73.0	75.8	70.6	72.2	78.8	72.4
Total micrococci.....	22.8	5.8	9.2	20.8	27.6	21.8	23.0	13.0	14.4	18.0	14.4	13.4	13.2	13.2	18.5
Quality of the stain.....	.....	.....	Good	Fair	Fair	Fair	Good	Poor	Fair	Fair	Good	Exc.	Exc.	Good	.....

\* One of these bacilli (0.2 per cent. of the bacteria counted) contained a spore.

were very rarely found; a single one is recorded in one count only, Subject C, May 23, 1908. As this is a unique observation, its significance must be regarded as doubtful. Very slender, undulating spirals we have classed as spirochetes. These measured from 3.5 to 6  $\mu$  in length; the thickness of the filament was about 0.25  $\mu$ , and the gross width of the coil from 0.5 to 0.9  $\mu$ . They were seen with great difficulty in unstained preparations but in a few instances where they happened to be especially numerous, they were seen and studied in the hanging drop. In the Gram stained preparations, and in the films stained with gentian violet for enumeration by the Eberle-Klein method, they were frequently observed. In some diarrheal stools these slender spirals were especially numerous. In these instances they appeared to be of the same kind as those ordinarily found in smaller numbers, but their specific identity with these is of course uncertain. The greatest number observed was 11.2 per cent of the fecal bacteria in Subject J on July 20, 1908. In many examinations none at all were found. The Gram positive bacilli varied from 13.0 per cent in Subject H, January 22, to 1.0 per cent in Subject G, July 29. Gram positive bacilli containing spores were seen from time to time in five of the 12 subjects. The greatest number in any one examination occurred in Subject E, on March 2, 0.8 per cent of the bacteria counted. The Gram positive cocci varied from 31.6 per cent in Subject C, July 27, to 0.4 per cent in Subject A, February 6. Free spores were seen in nearly every examination. Oval free spores varied in quantity from 8.6 per cent in Subject A, May 28, to 0.0 in several instances. Spherical free spores were most numerous in Subject I, January 12, 10 per cent of the bacteria counted. In a number of examinations these also were absent.

The Gram stained films were always predominantly Gram negative. The total Gram negative bacteria, not including spores, varied between 97.4 per cent in Subject J, February 27, and 63.4 per cent in Subject G, March 4. The total Gram positive bacteria, including those containing spores, varied from 34.0 per cent in Subject C, July 27, to 1.4 per cent in Subject J, February 27. The total free spores was highest in Subject A, February 24, 13.6 per cent, and fell to 0.0 in Subject D, upon several occasions. This did not occur in any other subject however.

Gram negative bacilli composed the majority of the fecal bacteria in every case, varying from 91.0 per cent in Subject J, February 27, to 54.8 per cent in Subject B, February 13. The total micrococci, disregarding the staining reaction, varied from 39.4 per cent in Subject H, January 6, to 5.0 per cent in Subject C, March 23.

These data are summarized in Table 7, where the maxima, minima, and the means for each kind of bacteria for each subject, and the maximum, minimum, and mean of all the examinations are given.

#### SUMMARY. (PART I. DIRECT EXAMINATIONS.)

1. A homogeneous suspension of the bacteria of adult human feces may be readily prepared by making it sufficiently dilute, 1:100.

2. In such a suspension the bacteria can be counted microscopically by an experienced observer, with a fair degree of accuracy.

3. The gravimetric method of Strasburger for determining the quantity of fecal bacteria is more time-consuming, but the method is capable of a higher degree of accuracy than the enumeration procedures. The separation of the bacteria should be done by fractional sedimentation in a high-speed centrifuge, and requires careful work at every step.

4. In the case of normal adult men, eating an ordinary mixed diet, the average number of fecal bacteria excreted daily is about  $33 \times 10^{12}$ ; the average daily bacterial dry substance about 5.34 gm., and the daily bacterial nitrogen 0.585 gm., making up 46.3 per cent of the total fecal nitrogen. There is considerable individual variation in the average quantity of fecal bacteria even in persons taking the same diet.

5. The results of enumeration do not correspond accurately with the results of gravimetric determination of the bacteria under different conditions. The fecal bacteria as individuals contain an amount of dry substance subject to considerable variation.

6. The bacteria of the adult human feces are Gram negative for the most part, about 70 per cent of all the bacteria being Gram negative bacilli. Gram positive rods are constantly present.

7. It is possible to recognize microscopically a number of morphologically different bacteria, present in such numbers that they must have resulted from multiplication in the intestine. A quantity of bacteria sufficient to yield 0.5 gm. or even 0.1 gm. of dry bacterial sub-

TABLE 7.  
SUMMARY OF DIFFERENTIAL COUNTS OF FECAL BACTERIA.

	Subject A	Subject B	Subject C	Subject D	Subject E	Subject F	Subject G	Subject H	Subject I	Subject J	Subject K	Subject L	All Subjects
<i>Bacillus coli</i> type.....	62.6 4.4 32.8	44.4 30.1 31.6	67.0 48.2 33.4	67.6 45.8 28.4	63.2 43.6 19.0	53.4 44.0 37.8	63.2 45.1 30.0	58.4 47.0 32.8	60.8 40.2 34.0	68.2 45.8 31.8	58.0 47.1 37.4	81.6 52.0 40.0	81.6 45.9 19.0
Other negative rods.....	37.8 27.2 12.3	36.6 26.7 17.0	35.2 25.6 8.2	40.0 24.0 2.0	46.2 31.3 8.2	32.4 22.0 14.0	32.3 25.0 10.0	35.4 24.2 0.4	44.4 25.4 3.0	40.0 27.6 3.2	32.4 22.2 17.4	27.2 10.5 7.6	46.2 25.3 0.4
Negative cocci.....	12.8 5.6 1.4	26.2 10.8 2.2	10.8 5.2 1.2	22.4 9.0 3.0	13.2 7.6 3.8	21.4 10.7 3.8	11.8 7.5 3.2	36.8 11.2 3.6	10.0 6.0 1.4	12.8 6.6 1.2	19.2 10.5 3.4	20.6 9.3 0.8	36.8 8.4 0.8
Negative spirilla.....	0.0 0.0 0.0	0.0 0.0 0.0	0.2 0.0 0.0	0.0 0.0 0.0	0.0 0.0 0.0	0.0 0.0 0.0	0.0 0.0 0.0	0.0 0.0 0.0	0.0 0.0 0.0	0.0 0.0 0.0	0.0 0.0 0.0	0.0 0.0 0.0	0.2 0.0 0.0
Negative spirochetes.....	3.2 0.4 0.0	3.8 0.8 0.0	5.2 1.8 0.0	7.6 2.4 0.0	2.0 0.5 0.0	1.0 0.3 0.0	3.4 1.1 0.2	1.8 0.6 0.0	1.4 0.4 0.0	11.2 2.8 0.0	9.4 4.3 0.0	1.8 0.5 0.0	11.2 1.1 0.0
Gram positive rods.....	11.9 6.2 3.8	7.2 4.0 2.4	11.4 4.4 2.2	11.0 7.4 3.6	7.8 3.8 1.6	12.0 8.4 2.2	11.2 5.2 1.0	13.0 4.8 1.8	8.0 5.0 1.6	8.6 4.6 1.4	11.8 7.0 3.2	9.8 7.1 3.2	13.0 5.7 1.6
Positive rods with spores.....	0.2 0.0 0.0	0.2 0.0 0.0	0.0 0.0 0.0	0.0 0.0 0.0	0.8 0.0 0.0	0.0 0.0 0.0	0.6 0.2 0.0	0.0 0.0 0.0	0.2 0.0 0.0	0.0 0.0 0.0	0.0 0.0 0.0	0.0 0.0 0.0	0.8 0.0 0.0



Positive cocci	Maximum.....	10.4	26.8	31.6	18.0	24.2	19.2	26.0	18.4	17.2	18.2	12.8	16.0	31.6
	{ Mean.....	12.4	15.9	13.1	9.6	11.2	12.0	12.5	10.5	9.3	10.0	9.9	9.2	11.3
	{ Minimum.....	0.4	3.2	2.0	3.4	1.2	7.8	4.0	2.6	1.0	4.2	2.8	2.2	0.4
Oval free spores	Maximum.....	8.6	0.8	3.4	0.8	2.2	1.8	4.4	3.0	7.0	4.8	2.2	1.0	8.6
	{ Mean.....	4.1	0.4	1.3	0.4	1.3	0.9	2.4	1.4	2.4	1.7	0.7	0.6	1.4
	{ Minimum.....	0.0	0.0	0.3	0.0	0.4	0.2	1.0	0.0	0.6	0.4	0.0	0.2	0.0
Spherical free spores	Maximum.....	8.6	1.2	2.2	2.0	2.8	1.4	4.4	0.8	10.0	3.0	7.2	2.0	10.0
	{ Mean.....	1.7	0.3	0.4	0.5	0.7	0.8	1.2	0.3	1.4	0.9	1.3	0.9	0.9
	{ Minimum.....	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Total Gram negative	Maximum.....	89.6	91.4	93.4	90.4	93.8	87.4	93.0	95.6	88.8	97.4	90.2	92.8	97.4
	{ Mean.....	75.6	79.3	80.8	82.1	83.0	77.0	78.7	83.0	81.9	82.8	81.1	82.2	80.7
	{ Minimum.....	69.4	68.4	65.0	75.6	70.0	72.0	63.4	76.2	75.2	73.6	74.6	72.8	63.4
Total Gram positive	Maximum.....	26.0	30.8	34.0	26.4	28.4	20.0	33.2	28.8	23.8	25.4	23.4	25.8	34.0
	{ Mean.....	18.6	20.0	17.5	17.0	15.0	20.4	17.7	15.3	14.3	14.6	16.9	16.3	17.0
	{ Minimum.....	4.2	7.4	5.2	7.8	2.8	10.2	5.0	2.6	3.0	1.4	7.6	5.4	1.4
Total free spores	Maximum.....	13.6	1.6	5.6	2.2	5.0	2.4	7.0	3.2	13.2	5.0	7.8	2.6	13.6
	{ Mean.....	5.8*	0.7	1.7	0.9	2.0	1.7	3.6	1.7	3.8	2.0	2.0	1.5	2.3
	{ Minimum.....	0.2	0.2	0.5	0.0	0.8	0.4	1.7	0.0	1.0	0.8	0.6	0.0	0.0
Total negative rods	Maximum.....	80.2	78.8	87.0	77.4	88.0	78.4	85.2	84.8	86.2	91.0	78.0	89.2	91.0
	{ Mean.....	69.6	67.8	73.8	60.8	74.0	66.0	70.1	70.1	74.6	73.4	60.3	72.4	71.1
	{ Minimum.....	60.1	54.8	62.4	63.2	64.0	55.2	59.2	56.8	65.2	55.0	57.2	59.8	54.8
Total micrococci	Maximum.....	31.6	36.0	32.8	26.8	30.2	32.0	31.2	39.4	28.4	26.4	29.4	20.2	30.4
	{ Mean.....	18.0	25.0	18.3	10.5	18.8	22.7	19.0	21.7	16.2	16.7	20.5	18.5	19.7
	{ Minimum.....	8.2	14.2	5.0	12.2	8.2	14.8	10.6	10.4	3.4	6.0	8.4	5.8	3.4

\* Note in this connection that Subject A excreted the dried feces of all the twelve subjects.

stance can hardly have been taken in as such with the food. Therefore there are several species of bacteria whose normal habitat is the human intestine.

8. Free spores are almost constantly present in considerable numbers in the feces. They are more numerous in dry stools as a rule. Diarrheal stools, however, sometimes show a very large number of free spores.

9. Thin, flexible spirals are quite frequently present in normal stools, and under some conditions are very numerous.

10. The direct quantitative determinations of the fecal bacteria furnish evidence of the extent and nature of the bacterial growth in the intestine. This seems to be a delicate index of intestinal conditions.

In conclusion we wish to acknowledge the encouragement and assistance of Professor Grindley, Chief of the Laboratory of Physiological Chemistry, without which it would have been impossible to complete this work. We are also indebted to Professor Theobald Smith of Harvard University for valuable suggestions.

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# THE VIRULENCE OF OLD AND OF RECENT CULTURES OF *BACILLUS PESTIS*.\*

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THE virulence of *B. pestis* when grown for a long time upon artificial media is a subject about which the evidence is conflicting. Some writers state that the virulence of the organism is soon lost; others hold that it is maintained for a long period.

I have had the opportunity to determine the virulence of a number of old strains of *B. pestis* and to compare it with the virulence of cultures of the organism that have been isolated during the recent plague campaign in San Francisco.

*Source of cultures.*—In this work I have used the eight stock cultures of *B. pestis* at the Hygienic Laboratory of the Public Health and Marine Hospital Service, Washington, D. C.<sup>†</sup> Unfortunately no accurate history of the majority of these cultures was to be obtained. They had, with one exception, been at the Hygienic Laboratory for several years; some of them for many years. The culture designated "X" had been in stock only a few months. They were kept on agar in sealed tubes in a dark room at a constant temperature of about 16° C. Every three or four months the cultures were transplanted to a new tube of agar, grown for a few days in the incubator at 37° C., and after a good growth was obtained they were returned to the cold room. It could not be learned when the cultures were passed through animals; that is, when they were inoculated into animals, recovered by culture methods from the body of the animal after death, and then returned to the stock. **It is definitely known that this had not been done for at least eight months before I began this work,** and it is very improbable that they had been treated in this manner for at least two years preceding these experiments. Such histories as were obtainable of the old cultures that proved virulent are given in connection with the tables showing the influence of graduated doses of

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the organism. The cultures are called by the names of the places where they were isolated, Manila, Bombay, Jedda, New York, Glasgow, X<sub>5</sub> (unknown), Frisco (San Francisco), Reedy Island. The cultures Bombay and Frisco proved to be practically avirulent. It was not known exactly how long these cultures had been at the laboratory, but Culture Frisco was isolated during the first plague campaign in San Francisco (1900-1904).

We have complete and accurate information concerning the strains of the organisms isolated in San Francisco during the recent plague campaign. These cultures are named to correspond with the animal from which they were isolated and with a number in our laboratory records; Human No. 171, Rat No. 66, Rat No. 82, Squirrel No. 1. Their histories are given in connection with the same series of tables as the old cultures.

*Verification of cultures.*—All of the old cultures, as well as the ones isolated in San Francisco, were carefully tested culturally and found to agree with *B. pestis* in every particular. The lesions produced in rats and in guinea-pigs by Manila, New York, Glasgow, X, Jedda, Rat No. 66, Rat No. 82, Human No. 171, and Squirrel No. 1 were characteristic of plague in these animals. Reedy Island produced typical lesions of plague in guinea-pigs. Frisco and Bombay killed guinea-pigs only when the animals were given the culture intraperitoneally, and the lesions were generally not those of acute plague. These two strains sometimes gave rise to the lesions of subacute or chronic plague. The white rats inoculated with Reedy Island, Frisco, and Bombay did not die so that in the case of these three cultures we lacked the characteristic lesions in these animals as confirmatory evidence of the nature of the organisms. There is no reason, however, for doubting that these avirulent cultures are strains of the true *B. pestis*.

The question presented itself as to whether these cultures of low virulence might be examples of *B. pseudotuberculosis rodentium* (Pfeiffer), but after comparing them with several strains of the latter organism I am convinced that such is not the case. The culture Reedy Island is known to have been isolated from a case of human plague and it is fair to assume that the same is true of the cultures Frisco and Bombay. The eight old strains were tested culturally by Acting Assistant Surgeon W. B. Wherry, who confirmed my findings. Dr.

Wherry investigated the reactions of these cultures on a series of sugar broths and they were found to give the fermentative reactions of *B. pestis*. He has kindly furnished me with the results of his work with the carbohydrates. "The cultures were grown at 36°-37° C. in +1 broth containing litmus and 1 per cent of various carbohydrates. They all produced acid (but no gas) from dextrose, levulose, galactose, maltose, and mannite, but did not ferment lactose, saccharose, nor inulin. The fermentative activity of the cultures was alike throughout—dextrose, levulose, and galactose being most actively fermented, mannite next, and maltose least. Throughout the series maltose was broken down only during the second 24 hours."

#### EXPLANATION OF TERMS USED IN TABLES.

*Day of death*.—The animals generally died a fraction of a day earlier than is shown in the tables, as all that died after 4 P. M. on any day were counted as dying on the succeeding day.

*Lesions*.—The lesions in guinea-pigs were regarded as those of **acute plague** when there was a brawny, bloody, or gelatinous local reaction, one or more caseous buboes surrounded by exudate which was usually bloody, an enlarged, friable spleen with many whitish granules; of **subacute plague**, when there was one or more caseous or purulent buboes with dense, tough capsules; with or without caseous or purulent foci in the spleen or in the lungs. The cases called subacute, or some of them, might with equal propriety be called chronic plague. The lesions of acute plague in the rat are a general subcutaneous injection, a bubo, a granular liver, an enlarged, firm spleen, and a pleural effusion. Smears were usually made for the purpose of demonstrating pest-like organisms. No record is made here of the results of the examination of smear preparations.

*B. pestis recovered*.—This refers to the recovery and identification of the organism by culture methods. Cultures were made from only one organ in the case of each animal and for convenience we generally made stroke cultures from the liver. The organism could have been recovered more frequently by the use of the plate method and by making cultures from more than one tissue, but on account of the limited time at my disposal this was not practicable. The identification of the organisms recovered was based on the character and appearance of the colonies on agar, the nature of the growth in broth, and the production of characteristic involution forms on 3 per cent salt agar. These three points are ample for establishing the identity of an organism as *B. pestis* when it is isolated from an animal having the typical gross lesions of plague.

The statement as to the generation of the organism used in the case of cultures Manila, New York, Glasgow, Bombay, Frisco, Jedda, X, and Reedy Island has reference to the generation on artificial media (agar) **after the cultures came into my possession at which time they had been grown for at least eight months on agar**. In the case of the cultures Human No. 171, Rat No. 66, Rat No. 82, and Squirrel No. 1 it refers to the generation after the original isolation from the naturally infected animal from which the culture was obtained.

The platinum loop used throughout the series of experiments was ellipsoid in shape, 1 mm. in its long diameter by 0.5 mm. in the short one. A calculation based



on the dimensions of this loop and upon the size of *B. pestis* indicated that it would take up about 260,000,000 of the organisms from an agar growth.

## VIRULENCE FOR GUINEA-PIGS

*Preliminary experiment.*—The first experiment was made by inoculating guinea-pigs by Kölles (cutaneous) method with a three-day-old agar culture, second generation. The results are shown in Table 1. One loopful of culture was used in each case.

TABLE 1.

Culture	Weight of Guinea-Pig	Day of Death	Lesions	<i>B. Pestis</i> Recovered	Remarks
Jedda.....	235 gm.	7	Acute plague	Yes	
Manila.....	260 gm.	6	" "	"	
New York.....		8	" "	Contaminated	
Glasgow.....	250 gm.	7	" "	Yes	
X.....		8	" "	"	
Reedy Island.....	245 gm.	7	" "	No growth	
Bombay.....			None	" "	Killed 21st day
Frisco.....			"	" "	" " "

*Influence of size of dose.*—The results obtained with old cultures that were supposed to require large doses subcutaneously to bring about a fatal infection were so surprising that it seemed desirable to investigate the subject further.

In each case a 24-hour agar culture was used; second generation. One guinea-pig was inoculated by the cutaneous method with one loopful of a suspension of the culture in physiological salt solution. The suspension was made to approximate the turbidity of a 24-hour broth typhoid culture. The other animal was given subcutaneously an entire agar culture suspended in salt solution. These quantities are obviously very inexact, but the latter dose was many hundred times the size of the former. In the table the first animal (A) under the head of each culture received the smaller dose; the second (B) received the larger dose.

TABLE 2.

Culture	Weight of Guinea-Pig	Day of Death	Lesions	<i>B. Pestis</i> Recovered	Remarks
Jedda..... { A	658 gm.	5	Acute plague	Yes	
{ B	510 gm.	3	" "	"	
Manila..... { A	495 gm.	8	" "	"	
{ B	490 gm.	7	" "	"	
New York..... { A	345 gm.	7	" "	"	
{ B	337 gm.	5	" "	"	
Glasgow..... { A	400 gm.	6	" "	"	
{ B	400 gm.	5	" "	"	
X..... { A	430 gm.	11	Subacute "	"	
{ B	407 gm.	4	Acute "	"	
Reedy Island..... { A	470 gm.		Subacute "	No	Killed 25th day
{ B	360 gm.		Enl'g'd Glands	"	" " "
Bombay..... { A	374 gm.		None	"	" 17th "
{ B	440 gm.		Abscess at site	"	" 14th "
Frisco..... { A	420 gm.		None	"	" 14th "
{ B	433 gm.		Abscess at site	"	" 14th "

The animals receiving the larger dose died earlier than the others, but except in the case of Culture X, the difference was not striking.

*Passage through a guinea-pig.*—The next series was undertaken for the purpose of ascertaining whether there was any change in the virulence of the organism due to its passage through a guinea-pig.

The first animal (A) under each culture was inoculated by the cutaneous method directly from the spleen of the animal in the preceding experiment that had been given an agar slant of culture subcutaneously. The control (B) was inoculated in the same manner (cutaneously) but with one loopful of a suspension of a 24-hour agar culture of approximately the turbidity of a 24-hour typhoid culture in broth. The culture was the second generation and was 24 hours old.

As the animals inoculated with Reedy Island, Bombay, and Frisco in the preceding experiment did not die they could not be made a part of the present experiment.

TABLE 3.

Culture	Weight of Guinea-Pig	Day of Death	Lesions	<i>B. Pestis</i> Recovered	Remarks
Jedda.....	A 345 gm. B 371 gm.	7 5	Acute plague	Contaminated Yes	Control
Manila.....	A 389 gm. B 384 gm.	not recorded 16	" " Subacute "	" No growth	Control—Death probably due to pneumonia
New York.....	A 322 gm. B* 345 gm.	9 7	Acute plague	Yes	Control
Glasgow.....	A 365 gm. B 400 gm.	7 8	" "	"	"
X.....	A 311 gm. B* 430 gm.	5 11	" " Subacute "	"	"

\* Data as to these guinea-pigs carried from preceding table to provide controls.

It will be observed that in the case of the cultures Jedda and New York the animals inoculated from the culture died earlier than did those inoculated from the spleen; on the other hand in the case of the cultures Glasgow and X those inoculated from the spleen died earlier than those inoculated from the culture. By an oversight the day of the death of the guinea-pig Manila A was not recorded.

*Graduated doses of culture.*—In the foregoing experiment there was practically no attempt made to give an accurate dose of cultures. In the following series we attempted to secure as high a degree of accuracy in dosage as was possible. Agar cultures 48 hours old were used in each case and the entire series of guinea-pigs used for the six old cultures was inoculated in the same afternoon, July 10, 1908. The procedure was as follows:

Normal salt solution (6.5 gm. per liter) was used in making the dilutions. Great care was exercised to break up the culture and make as perfect a suspension as possible. In making the inoculations the weakest dilution (0.000 001 of a loop) was injected first and the others in succession, using the strongest last. This was done to avoid the necessity of using a separate syringe for each dilution or of sterilizing the syringe after each inoculation. The error of carrying over in the syringe a small amount of the weaker dilution could not be ignored. The syringe was, of course, sterilized whenever the inoculation of a series (one strain) was completed. The dilutions were made of such strength as to make it convenient to use 1 c.c. of the suspension in each case. Each dilution after the first was made by mixing 9 c.c. of the normal salt solution with one c.c. of the next strongest dilution. The object was to make the injection subcutaneously into the abdominal wall, but on account of faulty technique the culture was given intraperitoneally in several instances. When this occurred it is so stated in the table.

There are, of course, very evident sources of error in work of this nature. The loop does not take up exactly the same volume of culture each time; sometimes some of the solution will escape at the site of inoculation; the organism may not be uniformly distributed through the medium. A more important source of error than any of these is the varying resistance of different animals. This is well shown in some of the tables.

For purposes of comparison I used four plague cultures isolated in San Francisco; one from a human case, two from rats, and the last from a squirrel. These four cultures had been isolated directly from the tissues of a human being and the lower animals: i. e., they had never been passed through a laboratory animal. The four last-named cultures were inoculated into animals on different days so that the results are not strictly comparable as they are in the case of the old cultures, but it is not believed that any material source of error existed on that account.

*Jedda*.—Nothing is known about this culture beyond the fact that it has been at the laboratory for at least eight years. The culture used was the fourth generation on agar. A 48-hour growth was used.

TABLE 4.

Weight of Guinea-Pig	Dose and Mode of Inoculation	Day of Death	Lesions	<i>B. Pestis</i> Recovered	Remarks
349 gm.	(subcutaneously)				
420 gm.	0.01 loop	2	Acute plague	Yes	Intraperitoneal
387 gm.	0.001 "	8	" "	"	
432 gm.	0.0001 "	13	Subacute "	No growth	
410 gm.	0.00001 "	8	Acute "	Yes	
	0.000001 "	5	" "	"	Intraperitoneal

*Manila.*—This culture was isolated in April, 1904, and was therefore four years old at the time these inoculations were made but there is no history as to what was the original source of the culture. The culture was the fourth generation on agar. A 48-hour-old growth was used.

TABLE 5.

Weight of Guinea-Pig	Dose and Mode of Inoculation	Day of Death	Lesions	<i>B. Pestis</i> Recovered	Remarks
	(subcutaneously)				
312 gm.	.01 loop	10	Acute plague	Yes	
438 gm.	.001 "	9	" "	"	
352 gm.	.0001 "	8	" "	"	
424 gm.	.00001 "	11	Subacute "	"	
333 gm.	.000001 "	12	Acute "	No growth	

*New York.*—This culture was isolated by Passed Assistant Surgeon John F. Anderson, P. H. and M. H. S., Assistant Director of the Hygienic Laboratory in the early part of 1904 and was therefore four years old when used. The culture was isolated from a guinea-pig inoculated from a case of human plague at the New York quarantine. The culture was the fourth generation on agar. A 48-hour-old growth was used.

TABLE 6.

Weight of Guinea-Pig	Dose and Mode of Inoculation	Day of Death	Lesions	<i>B. Pestis</i> Recovered	Remarks
	(subcutaneously)				
332 gm.	.01 loop	7	Acute plague	Yes	Intraperitoneal
306 gm.	.001 "	6	" "	"	
277 gm.	.0001 "	7	" "	"	
300 gm.	.00001 "	8	" "	"	
345 gm.	.000001 "	8	" "	"	Intraperitoneal

*Glasgow.*—No history is obtainable, but the culture is known to be at least four or five years old. The culture was the fourth generation on agar. A 48-hour-old growth was used.

TABLE 7.

Weight of Guinea-Pig	Dose and Mode of Inoculation	Day of Death	Lesions	<i>B. Pestis</i> Recovered	Remarks
	(subcutaneously)				
312 gm.	.01 loop	6	Acute plague	Yes	
432 gm.	.001 "	6	" "	"	
370 gm.	.0001 "	6	" "	"	
306 gm.	.00001 "	8	" "	"	
320 gm.	.000001 "	14	Subacute "	No growth	

*Culture X.*—This culture has been carried for about one year on artificial media. Nothing is known of its origin except that it was isolated from the spleen of a guinea-pig. The culture used was the sixth generation on agar. A 48-hour-old growth was used.

TABLE 8.

Weight of Guinea-Pig	Dose and Mode of Inoculation	Day of Death	Lesions	<i>B. Pestis</i> Recovered	Remarks
280 gm.	(subcutaneously) .01 loop	5	Acute plague	Yes	
295 gm.	.001 "	7	" "	"	
290 gm.	.0001 "	7	" "	"	
290 gm.	.00001 "	6	" "	"	
328 gm.	.000001 "	7	" "	"	

*Reedy Island.*—This culture was isolated in May, 1906, by P. A. Surgeon John F. Anderson from a case of human plague on a vessel at the Reedy Island Quarantine Station. The culture was obtained from a guinea-pig inoculated from the case. The culture was the fourth generation on agar. A 48-hour-old growth was used.

TABLE 9.

Weight of Guinea-Pig	Dose and Mode of Inoculation	Day of Death	Lesions	<i>B. Pestis</i> Recovered	Remarks
325 gm.	(subcutaneously) 1 slant	5	Acute plague	Contaminated	
365 gm.	1 loop	5	" "	"	
314 gm.	.1 "	8	" "	Yes	
416 gm.	.01 "	14	Subacute "	Contaminated	
355 gm.	.001 "	10	" "	No growth	
300 gm.	.0001 "	12	" "	" "	

*Human No. 171.*—This culture was isolated by routine methods by Acting Assistant Surgeon W. B. Wherry directly from the liver of a case of human plague which died in San Francisco in the early part of November, 1907. The culture used was the second generation on agar and was 48 hours old. The animals were inoculated nine months after the original isolation of the culture.

TABLE 10.

August 3, 1908.

Weight of Guinea-Pig	Dose and Mode of Inoculation	Day of Death	Lesions	<i>B. Pestis</i> Recovered	Remarks
263 gm.	(cutaneously) 1 loop	2	Early plague	Cultures not made	Pneumonia
298 gm.	(subcutaneously) 1 loop	2	Early plague	Cultures not made	Pneumonia
253 gm.	.01 "	4	Acute "	Yes	
318 gm.	.001 "	7	" "	"	
354 gm.	.0001 "	5	" "	"	
262 gm.	.00001 "	6	" "	"	
322 gm.	.000001 "	5	" "	"	

The first two animals showed beginning lesions of plague, but a pneumonia, probably due to a streptococcus, was the immediate cause of death.

*Squirrel No. 1.*—This culture was isolated in pure culture directly from the lung of the first plague-infected squirrel found in California. The culture used was the second generation and was a 72-hour agar growth. The culture was isolated six days before the inoculations were made.

TABLE 11.

August 12, 1908.

Weight of Guinea-Pig	Dose and Mode of Inoculation	Day of Death	Lesions	<i>B. Pestis</i> Recovered	Remarks
277 gm.	(cutaneously) 1 loop	4	Acute plague	Yes	
322 gm.	(subcutaneously) .01 loop	5	Acute plague	Yes	
279 gm.	.001 "	5	" "	"	
331 gm.	.0001 "	8	" "	"	
343 gm.	.00001 "	5	" "	"	Intraperitoneal
326 gm.	.000001 "	7	" "	No growth	

*Rat No. 66.*—This culture I isolated directly from the liver of natural plague rat No. 66 (new series) in San Francisco. The culture used was the fourth generation on agar and was 48 hours old. The animals were inoculated 41 days after the original isolation of the organism.

TABLE 12.

August 10, 1908.

Weight of Guinea-Pig	Dose and Mode of Inoculation	Day of Death	Lesions	<i>B. Pestis</i> Recovered	Remarks
350 gm.	(cutaneously) 1 loop	5	Acute plague	Yes	
425 gm.	(subcutaneously) .01 loop	3	Acute plague	Yes	Intraperitoneal
481 gm.	.001 "	7	" "	"	
349 gm.	.0001 "	6	" "	"	
420 gm.	.00001 "	7	" "	"	
465 gm.	.000001 "	16	" "	"	
317 gm.	.0000001 "	8	" "	"	

Owing to an error in making the dilutions one was made of one ten-millionth part of a loopful of the culture. This animal died eight days earlier than did the one receiving ten times as much culture.

*Rat No. 82.*—This culture was isolated from the liver of plague rat No. 82 (new series) at San Francisco. It was of rather more than ordinary interest as it was isolated apparently at or near the end of the epizootic in this city, the last previous case of rat plague having been detected 85 days prior to this one. This culture as is shown by the

TABLE 13.

Weight of Guinea-Pig	Dose and Mode of Inoculation	Day of Death	Lesions	<i>B. Pestis</i> Recovered	Remarks
326 gm.	(cutaneously) 1 loop	5	Acute plague	Yes	
293 gm.	(subcutaneously) .01 loop	4	Acute plague	Yes	
320 gm.	.001 "	10	Subacute	"	
251 gm.	.0001 "	4	Acute	"	
278 gm.	.00001 "	9	Subacute	"	
	.000001 "				Cultures negative, but spleen gave rise to typical acute plague in a guinea-pig from which <i>B. pestis</i> was recovered.



tables is quite virulent for guinea-pigs and white rats. There is, however, some evidence that it is less virulent for wild rats than are the other cultures isolated here. My work has not progressed far enough to enable me to speak definitely on this point. The culture used was a second generation 24 hours old and the inoculations were made 12 days after the original isolation of the culture.

The following compilation from the preceding tables shows the day of death of guinea-pigs inoculated with different doses of the several strains used.

TABLE 14.

	DOSE AND MODE OF INOCULATION							Average
	1 loop cutaneously	0.01 loop subcutaneously	0.001 loop subcutaneously	0.0001 loop subcutaneously	0.00001 loop subcutaneously	0.000001 loop subcutaneously	1 culture subcutaneously	
OLD CULTURES:								
Jedda.....	7	22	8	13	8	25	3	7.8
Manila.....	6	10	9	8	11	12	7	9.0
New York.....	8	27	6	7	8	28	5	6.8
Glasgow.....	7	6	6	6	8	14	5	7.4
X.....	7	5	7	7	6	7	4	6.3
Reedy Island.....	7	14	10	12				10.7
Average.....	7.2	7.0	7.2	8.2	8.2	11	4.8	7.5
NEW CULTURES:								
Rat No. 66.....	5	23	7	6	7	16		8.2
Squirrel No. 1.....	4	5	5	8	25	7		5.8
Human No. 171.....	5	4	7	5	6	5		5.4
Rat No. 82.....	5	4	10	4		9		6.4
Average.....	4.6	4.3	7.25	5.75	6.5	9.25		6.4

NOTE.—(a). Intraperitoneal not counted in making averages. (b). Reedy Island omitted in calculating averages at foot of columns as this culture was clearly of reduced virulence.

## VIRULENCE FOR WHITE RATS.

The next experiment was made with white rats. Full-grown animals were used. In each case the animal was inoculated by the cutaneous method with one loopful of three-day-old agar culture, third generation. The records of Human No. 171, Rat No. 66, Squirrel No. 1, Rat No. 82 are given as controls though they were not inoculated at the same time as were the other eight.

TABLE 15.

Culture	Day of Death	Lesions	<i>B. Pestis</i> Recovered	Remarks
Jedda.....	4	Acute plague	Yes	
Manila.....	4	" "	"	
New York.....	4	" "	Cultures not made	
Glasgow.....	4	" "	Yes	
X.....	5	" "	"	
Reedy Island.....	3	" "	"	
Bombay.....		None	No	Killed 13th day
Frisco.....		"	"	" 8th "
Human 171.....	6	Acute plague	"	" 8th "
Rat 66.....	4	" "	Cultures not made	Controls
Squirrel 1.....	4	" "	Yes	
Rat 82.....	4	" "	"	
	3	" "	"	

It is evident that the five old cultures that were constantly virulent for guinea-pigs were also quite virulent for white rats. The four cultures isolated in San Francisco were all fully virulent for the white rats.

#### SUMMARY.

These experiments demonstrate that of eight cultures of *B. pestis* that have been carried on artificial media for long periods five (5) Jedda, Manila, Glasgow, New York, X, are highly virulent, all of the animals inoculated with these cultures having died. One culture, Reedy Island, is not constantly lethal for animals. Two of these cultures, Frisco and Bombay, are practically avirulent.

The cultures isolated in San Francisco in the recent epidemic, Rat No. 66, Rat No. 82, Human No. 171, and Squirrel No. 1, are highly virulent.

The size of the dose and the mode of administration of virulent cultures (cutaneously or subcutaneously) have no marked influence upon the length of time an animal will live after inoculation. In the last statement an exception must be made in the case of such a colossal dose as a whole agar culture which generally kills earlier than the smaller doses, but in the case of such a large dose it is not improbable that an intoxication, owing to the large mass of bacilli introduced, plays a part.

Varying resistance of different guinea-pigs is of more importance than the dose of culture.

Passing the culture through one guinea-pig has no appreciable influence in raising the virulence.

These statements as to virulence refer only to guinea-pigs and white rats. Work is now in progress to determine the virulence of these cultures for wild rats (*Mus norvegicus*).

# AN EXPERIMENTAL STUDY OF JOINT AFFECTIONS INDUCED BY THE TYPHOID BACILLUS.\*

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AFFECTIONS of the joints, though not a rarity, are not excessively common during the course of typhoid fever. Despite their relative infrequency, certain clinical and pathological characters combine to render them of special interest. Among these points are the varied physical and bacteriological nature of the effusion, spontaneous dislocation, and the usually favorable, or at least non-fatal, course of the complication. This last fact largely accounts for our unsatisfactory knowledge concerning the pathology of these lesions.

Literature on both the clinical and experimental aspects of the subject is not conclusive. One point that was formerly disputed should now be regarded as definitely settled, namely, that the typhoid bacillus, even in the joints, is a pus-producing organism. This statement is not vitiated by the finding in some instances of sterile fluid in those cavities during the course of what clinically appeared to be a certain typhoid arthritis, as such finding is readily explained by experimental cases; the typhoid bacillus after producing the lesion dies out. Further, the cases in which the typhoid bacillus has been found in the pus of infected joints, although bacteriologic examination has too seldom been made, are sufficiently numerous to demonstrate the pyogenic properties of that organism.

Johnson<sup>1</sup> describes a case occurring in a woman of 25 years. During convalescence from typhoid fever her right wrist became swollen and painful. Pus aspirated from the joint contained a pure culture of the typhoid bacillus. Martin and Robertson<sup>2</sup> record a case in a man of 34. Pus aspirated from the fluctuating tumor about the wrist joint showed a pure culture of the typhoid bacillus. A second aspiration two weeks later showed only a small quantity of bloody pus.

Spontaneous dislocation of the hip is one of the most puzzling complications of typhoid fever. Ardisson<sup>3</sup> found that this disease was responsible for 33 of the 84 reported cases of this lesion; the other most frequent causes were polyarticular rheumatism with 23, and scarlet fever with 11 cases. Ardisson discusses at length the three leading theories of the cause of luxation, namely, (1) accumulation of fluid in the

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<sup>2</sup> *Montreal Med. Journal*, 1804, 23, p. 888.

<sup>1</sup> *Ann. of Surg.*, 1900, 32, p. 135.

<sup>3</sup> Thèse, Montpellier, 1903.

joint; (2) tissue proliferation in the acetabulum; and (3) the muscular theory. As before stated, this condition almost always terminates favorably and the pathology is therefore but little understood.

Graff<sup>1</sup> says that little is known of the mechanism of spontaneous luxation. Petit believed that an acute hydrarthrosis distended the capsule and ligaments. Verneuil and Reclus held that muscle pull plays a rôle and Hunter that the joint is dilated by an effusion of pus. From numerous Röntgen-ray studies of spontaneous dislocation after typhoid, puerperal sepsis, and acute osteomyelitis, Graff believes that in all cases the upper part of the acetabulum flattens, in part disappears, no actual destructive process, however, being demonstrable. The effusion in the joint is serofibrinous and without capsular dilatation leads to inflammatory softening of the upper acetabular rim. Pressure upon this by the head of the femur leads to atrophy and flattening so that the head slowly moves above and posterior.

Freeman<sup>2</sup> reports a case of spontaneous dislocation of the hip in a girl of 10 years. He says that most writers agree that the prime factor in dislocation is overdistension of the joint by fluid. The ligamentum teres is not necessarily ruptured, Moore having experimentally demonstrated that it may be sufficiently elongated by an intraarticular effusion to allow of complete dislocation without loss of continuity.

Kummer<sup>3</sup> says regarding the content of the joint in cases of spontaneous dislocation of the hip in connection with acute infectious diseases, that in 29 cases in which the presence of arthritis was known before dislocation occurred, 22 were serous or serofibrinous and three purulent. Kummer adds two typhoid cases of his own but does not give the bacteriology of any of the 51 cases of spontaneous dislocation of which he presents brief notes.

I have found but two reports that are at all definite upon the pathology of spontaneous dislocation, and one of these is valuable largely for its negative findings. Both were old operative cases and hence throw no light on the early stages of the joint affection.

Rawdon<sup>4</sup> describes a case occurring in a girl of eight years. He first saw the patient in June, the history being that during an attack of typhoid fever which lasted from February to May, she complained of pain in the right thigh and knee but not in the hip; later, deformity of the thigh developed. Rawdon operated, removing the head of the femur. The head showed no notable absorption. Synovial membrane was not demonstrable. The cartilage was in general intact, showing only a little absorption around the circumference at its junction with the neck. The bony structure of the head and neck was absolutely normal and the surrounding tissues were notably healthy.

Hübener<sup>5</sup> reports the case of a girl of 14 years, admitted to the hospital April 1. The preceding September she had an attack of typhoid fever with a short recurrence on the 31st day. Two months afterward the right hip became affected. The foot and knee previously swelled but soon subsided under treatment. Pain in the hip kept the child in bed for a time and then the hip was found to be out of joint. A skiagraph showed dislocation of the femur, the acetabulum being still well formed with a sharp

<sup>1</sup> *Centralbl. f. Chir.*, 1901, 28, p. 1243.

<sup>4</sup> *Liverpool Med. Chir. Jour.*, 1882, 2, p. 22.

<sup>2</sup> *Med. News*, 1895, p. 452.

<sup>5</sup> *Mitteil. a. d. Grenzgebiet. d. Med. u. Chir.*, 2, H. 5.

<sup>3</sup> *Revue de Chir.*, 1898, 18, pp. 55, 111, 319, 632.

border. The head of the femur was loose at the epiphyseal line. Operation revealed the acetabulum empty and covered by slight masses of granulation tissue. The head of the femur was free and lay behind and above the acetabulum imbedded in a moderate amount of granulation tissue. The head of the bone and the new tissue were removed. In this tissue were typhoid bacilli. The patient died but autopsy was not permitted. Hübener believes there was exudate in the joint before dislocation occurred.

As with the clinical, so with the experimental studies of typhoid joint affections. But little work along this line has been done and the findings are therefore not conclusive. Investigations have been made chiefly upon rabbits, as, though those animals are quite refractory to the typhoid bacillus, they are in many ways the most suitable for experimental work.

Orloff<sup>1</sup> found that the effusion in joints after the injection of typhoid bacilli may be serous, seropurulent, or purulent, the first and second being most frequent. He injected 13 rabbits in the knee joint, at the same time injecting into the opposite joint sterile water or broth, according as he had employed an emulsion of agar growth or broth culture of the bacillus. One-half to one cubic centimeter of the material was used. The joints were studied after periods of time ranging from 15 hours to 37 days.

Twenty-four hours after injection the joints were swollen and the subcutaneous tissues edematous. In some instances the whole lower leg showed edema, this probably being partly due to the 5 per cent carbolic acid solution used to disinfect the leg. Removal of the skin during the first 12 to 24 hours revealed dilated vessels, this also being true of the synovial membrane; at times there was actual hemorrhage. In the cases investigated later the hemorrhage was slight. In the joints, especially the upper recess, was a thick, tough, more or less cloudy fluid not seldom in large quantities. Joints examined later contained opaque, white, moderately hard, kernel-like masses and the fluid was more turbid and seropurulent in type. In from 24 to 37 days the fluid was still more thick and adherent to the synovial membrane. In the fluid of the first day, also later, were pus cells in small or large numbers. Typhoid bacilli were present during the first two days; after eight or nine days they were with one exception absent; in that one instance they were found on the 13th day. In the control knees the injected fluid was entirely absorbed.

Orloff also injected typhoid bacilli into muscles, under periosteum, etc., in order to investigate the pus-producing properties of that organism. His résumé concerning the joint lesions was that there was produced a seropurulent, frequently thick, mucus-like exudate. He afterward repeated his experiments using sterilized cultures instead of living bacilli and obtained the same results, though the reaction was weaker. One of his conclusions was that the suppurative processes developing during typhoid fever, when the pus contains exclusively typhoid bacilli, are due to that organism alone and are not to be regarded as the consequence of a mixed infection of typhoid bacilli and puccini.

Arcoles<sup>2</sup> injected broth cultures of the typhoid bacillus into joints or veins of five young rabbits. He produced suppurative arthritis by direct injection into joints or by injuring joints after injecting bacilli into a vein. Bacilli were found in the effusion in the affected joints.

<sup>1</sup> *Centralbl. f. Bakt.*, 1800, 8, p. 366.

<sup>2</sup> Abs. in *Centralbl. f. Chir.* 1898, p. 266.



Florange<sup>1</sup> believes that typhoid arthritis comes often from lesion of the neighboring bone. The typhoid bacillus is constantly in the bone marrow of those suffering from typhoid fever and its pyogenic powers are well known. It may then invade a joint from the bone and thus be a propagation from an osseous infection. Most often, he believes, there is not a typhoid infection, properly so called, of the joint, but simply an irritation of the neighboring parts caused by an inflamed epiphysis; the articular effusion is sterile. The epiphyseal osteitis is here the principal lesion, the arthritis being secondary and less important. Florange's contention is partially supported by the experiments of Klemm,<sup>2</sup> who injected eight rabbits in an ear vein and afterward found in the bone marrow of five of them a pure culture of the typhoid bacillus, though arthritis was not induced. Pus was found in none of the cases.

My object in carrying out the experimental study here recorded was to add to our knowledge of the action of the typhoid bacillus in joints, especially, if possible, after causing their presence in those cavities by means other than direct injection.

The first set of experiments was made by using a broth growth of a stock culture of the typhoid bacillus of uncertain virulence. One c.c. of a 24, 48, 72-hour, or older, growth was injected directly into a knee joint and those structures examined at various periods later. In all of this series the skin over the site of injection was first washed with soap and water and then with a 5 per cent carbolic acid solution. When the joints were examined, spreads and inoculations were made from the joint content and in most instances tissue was fixed and finally studied in stained sections. The results can best be given in brief protocols of the individual animals. Spontaneous death as it occurred in some of the rabbits, with the exception of the one due to pneumonia, in these series of cases, was apparently due to the lesion produced or to the conditions of captivity; at least no anatomic changes pointing to other more definite causes were found.

RABBIT 1.—One c.c. 24-hour broth culture typhoid bacillus injected in knee joint. Next day joint red and much swollen, second day moderately swollen, third day essentially normal. (These early phenomena were common to all the animals of this series.) Rabbit chloroformed on seventh day. Joint moderately distended, motion less free than on opposite side. In joint is 0.5 c.c. thick, tenacious, grayish fluid. Synovial membrane lusterless. Microscopically, fluid contains numerous cells, 53 per cent polynuclear, 47 per cent mononuclear. Many of the latter are evidently endothelial cells and some of them contain polynuclear leukocytes in varied stages of disintegration. Sections through the cartilage of the femur show a covering of granular acidophilic detritus and leukocytes but no erosion. Inoculations from the joint were sterile.

RABBIT 2.—One c.c. 48-hour broth culture typhoid bacillus injected in knee

<sup>1</sup> Thèse de Paris, 1902.

<sup>2</sup> *Archiv f. klin. Chir.*, 1893, 46, p. 862.



joint. Animal killed on 10th day. Joint slightly swollen and motion less free. Practically no free fluid in joint cavity. Spreads from scrapings contain many cells, mononuclear 87 per cent, polynuclear 13 per cent. Tissues show no lesion. Inoculations from the joint remained sterile.

RABBIT 3.—One c.c. 72-hour broth culture typhoid bacillus injected in knee joint. Animal found dead on 10th day, the joint having been gnawed open by other rabbits. In the spreads from the content are great numbers of cells, approximately 65 per cent mononuclear, though degeneration is too advanced for an accurate count. Synovial membrane shows engorgement of vessels and infiltration by mononuclear cells; no erosion.

RABBIT 4.—One c.c. 72-hour broth culture typhoid bacillus injected in knee joint. On second day an unsuccessful attempt was made to withdraw fluid from the joint by means of a hypodermic needle. Rabbit chloroformed on 58th day. No erosion of tissues. Inoculations gave growth of unidentified cocci.

RABBIT 5.—One c.c. six-day broth culture typhoid bacillus injected in knee joint. Rabbit found dead on 33rd day. Joint not swollen and contains small quantity of tenacious, almost clear fluid. Vessel around joint slightly injected. No erosions. In spreads a few cells, all mononuclear, well preserved. Inoculations sterile.

RABBIT 6.—One c.c. 48-hour broth culture typhoid bacillus injected in knee joint. Rabbit killed on fourth day. Joint is moderately swollen and motion less free than on opposite side. Interior of joint essentially the same as in Rabbit 1. Spreads contain many cells, 88 per cent of which are polynuclear. No erosions.

RABBIT 7.—One c.c. 48-hour broth culture typhoid bacillus injected in knee joint. Rabbit found dead on 16th day. Apparently only normal amount of synovial fluid within joint. Spreads contain very few cells, many so degenerated that accurate differential count cannot be made. Tissues of joint show no definite lesion. Inoculation sterile.

RABBIT 8.—One c.c. six-day broth culture typhoid bacillus injected in knee joint. Animal killed on 45th day. No evident gross lesions. Few cells in spreads, all mononuclear and well preserved. Tissues show no lesion. Inoculation from joint sterile.

RABBIT 9.—One c.c. 24-hour broth culture typhoid bacillus injected in knee joint after it was roughly manipulated for one minute. Rabbit found dead on sixth day. Considerable thick, grayish fluid in joint. Synovial membrane grayish. In spreads are many cells, polynuclears 72 per cent. Tissues show no lesions. Inoculation gave pure culture of typhoid bacillus.

RABBIT 10.—One c.c. 24-hour broth culture typhoid bacillus injected in knee joint, which was then scraped with point of needle. Rabbit found dead next day. No free fluid in joint but a scanty, tenacious, almost clear material adheres to synovial membrane. Latter structure ecchymotic where injured by needle. In spreads are enormous numbers of cells, 98 per cent polynuclears. Inoculation from joint gave pure culture of typhoid bacillus.

The results of these experiments indicated that the bacilli employed were not sufficiently virulent to produce noteworthy lesions in the joint tissues, although an inflammatory exudate appeared in the cavity. They did show that at first the cells in the exudate were practically all polynuclear leukocytes, these after a few days giving

way to mononuclears. The latter were nearly all of the large or hyaline variety of lymphocyte or were endothelial cells, very few lymphoid cells being found. The endothelial cells were phagocytic for polynuclear leukocytes.

In the eight cases in which inoculations were made from the joint the typhoid bacillus was found twice, after the lapse of one and six days respectively. Five others were sterile at the end of 7, 10, 16, 33, and 45 days. One only, No. 4, contained bacteria other than the typhoid bacillus; the presence of cocci in this case is fully explained by the fact that on the second day an attempt was made to aspirate the joint.

In the second series of experiments, the typhoid bacilli employed were increased in virulence by repeated passage through guinea-pigs until one-tenth loop intraperitoneally was sufficient to kill a 200-gram pig within 18 hours. Emulsions of 24-hour agar growth in sterile water were used for injection. In order to avoid the irritating effect of carbolic acid solution, the skin over the joint was prepared by simply washing with alcohol. That this was sufficient precaution is shown by the fact that contamination not once occurred, inoculations from the joints always giving a pure culture of the typhoid bacillus or remaining sterile. The typhoid bacillus was identified during its preparation and in the cultures from joints by the fermentation, Conradi-Drigalski, and agglutination tests.

I began this series with the attempt to induce lesions of joints in some way other than by direct injection into them of bacilli. With this aim several methods were employed only to be abandoned because of anatomic or operative difficulties or because of lack of results. The 10 experiments in this group are as follows:

RABBIT 11.—One-tenth loop typhoid bacilli injected into left carotid artery and double ligature applied. Animal died in 13 days from double pneumonia and pleuritis. No joint lesions.

RABBIT 12.—Injected same as No. 11. Rabbit apparently sound at end of 57 days.

With several rabbits attempts were made, after the injection of bacilli into the circulation, to create by trauma or by interference with the circulation a *locus minoris resistentiae* in one or more of the joints, with the hope that in this way the bacilli might be induced to colonize therein. This object was not attained. In one animal a

fruitless attempt was then made to throw the bacilli into the circulation at a site nearer the joint than is the ear.

The idea of causing localization of bacilli in joints by way of the circulation was then abandoned and the remainder of the experiments confined to injecting bacilli directly into the joints. To produce a condition comparable to that in human beings with typhoid fever, if the bacilli lived, injections were made into the veins of four rabbits at different periods before the joints were inoculated.

RABBIT 13.—One-tenth loop typhoid bacilli injected into ear vein. One week later one loop bacilli injected into knee joint. On following day the joint was red and greatly swollen. Animal killed on seventh day when nearly dead. The injected joint was loose, that is, permitted unusual latitude of motion especially in the lateral direction. In the joint was a small quantity of soft, yellowish, granular material. This was in connection by small openings through the capsule with two periartritic abscesses. Spreads of the joint content show many endothelial cells, most of which contain numerous vacuoles. In addition are enormous numbers of leukocytes, at least three-fourths of which appear to be polynuclear though many are so degenerated that their type cannot be determined. A few threads of fibrin are present. Inoculation from the joint remained sterile.

RABBIT 14.—Injected in ear and joint same as No. 13. Animal killed on eighth day when nearly dead. Injected joint very loose, can be fully flexed readily and bent laterally to angle of 50 degrees. The quadriceps tendon can easily be thrown out of its groove by manipulating the joint; this cannot be done with the opposite leg. In the joint is a considerable quantity of yellow, moderately tenacious fluid. Over the surfaces of the joint and extending upward for some distance in the sheath of the quadriceps tendon is a yellowish, friable deposit resembling degenerated fibrin. In the posterior recess of the joint is 1 c.c. of thick, yellow pus.

The interarticular ligaments are destroyed, remaining only as indefinite masses of reddish-yellow debris covered by the described exudate. The semilunar and articular cartilages are not appreciably affected, either in color or consistency. In spreads from the joint content are many leukocytes, 95 per cent of which are polynuclears. In many only the nucleus remains. There is a moderate number of endothelial cells, with more or less fatty degeneration of the protoplasm. Inoculations from the joint gave a pure culture of the typhoid bacillus.

RABBIT 15.—One-tenth loop typhoid bacilli injected in ear vein and four days later one loop in third joint of hind leg. Animal found dead on 13th day. Pronounced serofibrinous pleuritis and pericarditis. Joint contains small quantity of tenacious, turbid fluid, in which are endothelial cells and moderate number of leukocytes, about half of the latter polynuclears.

RABBIT 16.—One-tenth loop typhoid bacilli injected in ear vein and three days later one loop in third joint of hind leg. Animal found dead on 47th day, the joint differing in no wise from the corresponding one on the opposite side.

The results in Rabbits 13 and 14 show clearly that a suppurative arthritis can be induced by the typhoid bacillus. Further, and this

is of special importance in connection with the problem of spontaneous luxation, is the fact that motion in the affected joints was of much greater latitude than is normal. This in No. 14 is readily explained by the destruction of the interarticular ligaments; in the other, changes in the capsule and surrounding tissues, with possibly some yielding of the ligaments, must be held accountable. That a suppurative arthritis is present as the underlying lesion in all, or even any, of the cases of spontaneous luxation in human beings cannot be said; in truth, the clinical history of most of them would indicate that such was not the case. Notwithstanding this, the fact that the typhoid bacillus can occasion such destruction of articular tissues as was found in Case 14 is of importance. This case is further of interest in that the typhoid bacillus was still living in the joint on the eighth day after injection.

In the remaining experiments of this series, typhoid bacilli were injected directly into the knee joint, this being the only expedient employed.

RABBIT 17.—One-half loop typhoid bacilli injected in knee joint about 4 P.M. Animal found dead next morning with no evidence of reaction in the joint. Spreads from the joint content contain numerous bacilli and occasional endothelial cells. Inoculation from the joint gave a pure culture of the typhoid bacillus.

RABBIT 18.—One loop typhoid bacilli injected in knee joint. Next day, joint red and greatly swollen. Animal killed on fourth day. Joint much swollen, containing a thick, tenacious, yellow fluid. In spreads are many cells, 93 per cent polynuclears, most of remainder endothelial cells. Many of both types show extensive degenerative changes. A few of the polynuclears contain bacilli. The joint tissues show no erosion. Inoculation from joint gave pure culture of the typhoid bacillus.

RABBIT 19.—One-half loop typhoid bacilli injected in knee joint. Next day, joint red, swollen, and tender. Third day rabbit sick, with profuse diarrhea. On sixth day was emaciated but fairly lively; was killed. In the swollen joint is considerable turbid fluid. This is especially true of the posterior part from which a mass of cheesy material extends into the surrounding tissues. Articular surfaces and ligaments show no gross lesion. Spreads contain enormous numbers of leukocytes, 95 per cent polynuclears, and many endothelial cells, the latter showing extensive fatty degeneration. Inoculation from joint proved sterile.

A piece of the muscle bordering the joint was prepared and sectioned. Sections including the joint capsule and attached tissue show on the former a mass of exudate consisting of leukocytes, fibrin, and red cells. A few endothelial cells are also present. The outer portions are thickly infiltrated by mononuclear cells, chiefly large with vesicular nuclei, among which are a few polynuclears. The muscle has for the larger part almost or entirely lost its cross striation. The fibers are in many instances partially hyaline and show pronounced longitudinal furrowing or actual separation into fibrils. At points the blood vessels are surrounded by mantles of mononuclear cells.

RABBIT 20.—One loop typhoid bacilli injected in knee joint. Next day joint much swollen and red. Animal found dead on eighth day, the joint being greatly swollen. In it is a large quantity of thick, yellow fluid exactly resembling pus as found in human beings. This permeates the entire joint, being beneath the semilunar cartilages. Adherent to the articular surfaces is a quite thick layer of grayish-yellow material undoubtedly fibrinous in character. The interarticular ligaments are covered by the exudate and the superficial portions are necrotic. The periarticular tissues are also infiltrated by pus. The articular cartilages are not eroded. Spreads from the joint content contain a great deal of fibrin and numerous leukocytes, many of which show pronounced degeneration; the great majority of those still identifiable are polynuclears. A few degenerated endothelial cells are present, as are also occasional bacilli. Inoculation from the joint contents, though only a few colonies grew, proved a pure culture of the typhoid bacillus.

Portions of the quadriceps tendon and of the adjoining muscle were fixed and sectioned. Microscopically the muscle is largely necrotic, these areas being closely packed with leukocytes. Most of these are polynuclears, though bordering the necrotic masses mononuclears predominate. The outlying portions of muscles not yet necrotic have lost their transverse striation. Between the fibers are many mononuclear cells and also numerous polynuclear eosinophiles. The inner surface of the tendon is also partly necrotic. In the remainder the fibers are separated by mononuclear leukocytes and here, as in the muscle, are polynuclear eosinophiles.

RABBIT 21.—One-half loop typhoid bacilli injected in knee joint which became red, swollen, and tender. Animal found dead on eighth day. The joint is swollen but its motion not freer than that of the one opposite. In the joint is a small quantity of thick, yellow fluid, which in the posterior pocket becomes caseous. The anterior interarticular ligament is covered by exudate and the superficial portion is slightly necrotic. Just posterior to the insertion of this ligament on the tibia is an area of erosion 2 mm. in diameter, in which jagged bone is exposed.<sup>1</sup> Spreads of the joint content contain many leukocytes, a large number degenerated but the great majority polynuclear. Endothelial cells, some showing fatty degeneration, are also present. Inoculations were not made.

Sections through the joint capsule and adherent tissues are densely infiltrated by mononuclear cells, many with large, partly vesicular nuclei. At points are masses of fibrin. The quadriceps muscle shows distention of blood vessels, collection of mononuclear leukocytes in the interstitial tissue, and hyaline degeneration of some of the fibers.

RABBIT 22.—One-half loop typhoid bacilli injected in knee joint which became red, swollen, and tender. Animal found dead on eighth day. The joint is very loose, especially permitting excessive lateral motion. In the joint is a large amount of grayish-yellow fluid. The anterior interarticular ligament is reduced to a few threadlike masses of reddish material covered by exudate. The internal borders of the semilunar cartilages are eroded and thinned; the articular cartilages are intact. Spreads of the joint fluid contain many leukocytes, 95 per cent polynuclears, showing but little degeneration, a moderate number of fatty endothelial cells, and numerous threads of fibrin. Inoculations were not made. Sections from the quadriceps muscle show extensive infiltration by mononuclear leukocytes, particularly in the vicinity of vessels. The muscle shows pronounced granular degeneration.

<sup>1</sup> Bone from this and other cases in which that structure was involved, or at least kept for investigation, was accidentally destroyed by fire and therefore microscopic studies of it could not be made.



RABBIT 23.—One-third loop typhoid bacilli injected in knee joint. Animal found dead on eighth day. Joint is swollen but not loose. In the joint is yellowish-gray exudate. The capsule anteriorly and the quadriceps muscle extending over and along the tendon are largely necrotic. The interarticular ligaments are covered by exudate but not otherwise appreciably affected. Spreads show essentially the same as in No. 22. Inoculation from joint sterile. Sections through the quadriceps muscle well above the exudate show at the tendon insertion a perivascular round-cell infiltration. Many of the fibers have lost their transverse striation and stain less deeply than do those of normal muscle.

RABBIT 24.—One-third loop typhoid bacilli injected in knee joint. Animal found dead on 10th day. In the greatly swollen joint is a large quantity of yellowish exudate. The interarticular ligaments, semilunar and articular cartilages show no gross lesions. In spreads of the exudate is a great deal of fibrin. The cells, though both poly- and mononuclear can be recognized, are too much degenerated to permit definite statements regarding their percentages. Inoculation from the joint gave a pure culture of the typhoid bacillus.

RABBIT 25.—One-third loop typhoid bacilli injected in knee joint. Animal found dead on 11th day. The joint is moderately swollen. Vessels of fascia around and for some distance above the joint are markedly injected. The joint capsule is also injected and at points anteriorly is much softened and easily torn. In the joint is a small quantity of yellowish fluid and in the posterior recess a collection of yellow, granular material. There is no erosion of ligaments or cartilages. In spreads of the joint fluid is a moderate number of leukocytes, many degenerated, the majority present being polynuclears. Inoculations from the joint gave a pure culture of the typhoid bacillus. Sections from the quadriceps muscle show slight granular degeneration of that structure.

RABBIT 26.—One loop typhoid bacilli injected in knee joint. Animal killed on 12th day. In the joint, which permits freer motion than that on the opposite side, is a small quantity of yellow, tenacious material. Extending into the surrounding tissues from posterior portion of joint is a cavity containing 1 c.c. thick yellow pus. In spreads of the joint fluid are a great number of leukocytes most of which are markedly degenerated but the majority are polynuclear in type. Inoculations from the joint proved sterile.

Sections from the muscles bounding the joint show in areas atrophy but most prominent is hyaline degeneration which here is very extensive. Entire fibers, in some instances several adjoining ones, have lost all structure and appear as a hyaline material staining very slightly by eosin. Such areas are widespread through the sections. Small areas of necrosis are also present, confined to the fibers alone. Some of the necrotic material stains very deeply by hematoxylin, suggesting the probability of calcareous deposits. Between these necrotic muscle fibers the interstitial tissue is very densely infiltrated by leukocytes, the majority mononuclear. In some of the sections the hyaline degeneration is very prominent and necrosis and leukocytic infiltration slight or entirely lacking.

RABBIT 27.—One-third loop typhoid bacilli injected in knee joint. Animal found dead on 14th day. Joint swollen with yellow discoloration above and below. The capsule is grayish-yellow and soft. In the joint is a small quantity of yellowish fluid with considerable granular material in the posterior part. The interarticular ligaments are covered with exudate but not eroded. Spreads of the joint fluid contain many degenerated leukocytes, a few endothelial cells, and threads of fibrin. Inoculations from the joint were sterile.



RABBIT 28.—One-third loop typhoid bacilli injected in knee joint. Animal found dead on 14th day. In the greatly swollen joint is a small quantity of yellow, tenacious fluid. Between the interarticular ligaments and extending laterally, the head of the tibia is bared and eroded over an area 0.5 cm. in diameter, presenting a rough, spiculated surface. The capsule is partly necrotic; the interarticular ligaments and joint cartilages are not eroded. Spreads from the joint fluid contain many leukocytes, a few fibrin threads, and a number of greatly degenerated endothelial cells. Inoculations from the joint were sterile. Sections of the muscles bordering the joint show necrosis and leukocytic infiltration as described under No. 26.

RABBIT 29.—One loop typhoid bacilli injected in knee joint. Animal found dead on 16th day. In the swollen joint is a moderate quantity of quite thick, yellow fluid. Beneath the semilunar cartilages is a thin layer of fibrinous exudate. In the middle of the head of the tibia, extending from the anterior margin to the interarticular ligament and to a small extent over the articular surfaces, is an almost circular area 1 cm. in diameter in which the bone is bared of its covering and is carious and jagged. From this point the anterior portion of the capsular ligament is necrotic and opens into an abscess which extends under the tendon and to the surrounding tissues. The capsule is also destroyed posteriorly and the joint cavity here connects with the surrounding abscess. On one of the articular facets of the femur the articular cartilage is superficially eroded over an area 3 by 8 mm. in extent. In spreads from the joint content are a few partly degenerated endothelial cells and an enormous number of leukocytes, 90 per cent of which are still recognizable as polynuclears. Inoculations from the joint proved sterile.

SUMMARY OF THE RESULTS IN THE 13 RABBITS, NOS. 17 TO 29 INCLUSIVE, IN WHICH DIRECT INJECTION OF TYPHOID BACILLI INTO A JOINT WAS THE METHOD EMPLOYED.

In all of the 12 animals which survived a sufficient length of time, a seropurulent or purulent exudate appeared in the joint. In three instances there was more or less extensive necrosis of interarticular ligaments. In one there was erosion of the semilunar cartilages. In three cases there was destruction of the overlying soft parts and erosion of the articular surface of bone, in each instance the head of the tibia being affected. In three cases the muscles bordering the joint showed hyaline degeneration, accompanied or not by areas of necrosis. Inoculations were made from the joint content in 11 of the 13 cases. The typhoid bacillus in pure culture was recovered in five instances, after the lapse of 1, 4, 8, 10, and 11 days respectively. The exudate was sterile in six cases, at the end of 6, 8, 12, 14, 14, and 16 days.

In order to verify Orloff's findings as regards the effect of dead bacilli in the joint, I used this material in two experiments. The source of the bacilli employed was guinea-pig 19, the same as that of the organism employed for many of the knee injections already described, hence of equal virulence. The suspension of bacilli was

placed before injection for one hour in an oven at 60°C.; inoculations made from this suspension at the time of injection proved it to be sterile.

RABBIT 30.—One loop typhoid bacilli killed by heating for one hour at 60° C. injected in knee joint. Next day the joint was red, swollen, and tender. Second day less swollen, third day normal in size and color. Animal found dead on the eighth day. The injected joint microscopically is exactly like the corresponding one of the opposite side. Spreads from the fluid contain a few well-preserved endothelial cells.

RABBIT 31.—Injected same as No. 30. Next day the joint was red, swollen almost as much as those in which living bacilli were used, and tender. Second day was less swollen, third day normal in appearance. Animal found dead on 25th day. Knee joints on the two sides absolutely alike.

These two experiments are of course insufficient upon which to base conclusions but the results in my cases are so opposed to those of Orloff as to be noteworthy. He obtained the same effect, except in lesser degree, from killed as from living bacteria. What my two animals would have shown had the joints been examined during the quite violent, but temporary, reaction of the first day or two after the injection is problematical, but Orloff's statement was based on a comparison with his other series which included periods of time up to 37 days.

Since cases of spontaneous luxation as a feature of typhoid joint affections in human beings are limited to the hip, I attempted finally, for the sake of better comparison, to induce lesions in that joint, instead of in the knee, in rabbits.

RABBITS 32 TO 38 INCLUSIVE.—Attempted injection of typhoid bacilli into hip joint. Owing to the depth of the joint and its anatomical structure, the capsule was not penetrated by the needle in any of the seven cases. The bacilli were deposited in the tissues overlying the joint or in the neighboring muscles.

These animals all died in from 4 to 14 days, presumably from the effects of abscesses or local necroses which developed at the site of injection. The muscles bordering these lesions show in stained sections hyaline degeneration and necrosis as already described in connection with a number of the knee cases. In one instance, in addition to these lesions, the bordering muscle shows quite marked increase of connective tissue, amounting to a distinct productive interstitial myositis.

In general the experiments described in this paper show clearly that the typhoid bacillus is capable of seriously damaging and even destroying the various elements entering into the formation of joints. Collectively, all the component parts of those structures—synovial membrane, interarticular ligaments, semilunar cartilages, articular cartilages, bone, the joint capsule—and the surrounding tissues, including the

muscle, were thus affected. Certain of these points, to the best of my knowledge, had not previously been demonstrated and they consequently throw some light on those phases of the pathology of this affection.

Just how far it is safe to apply these findings in animals to the interpretation of typhoid joint affections in human beings is difficult to say. I believe we are justified in considering that all the destructive effects here enumerated are at least possible in human joints. There at once arises the objection that the bacilli experimentally were injected directly into the joints while in man they enter those structures, if they are present, directly or indirectly by way of the blood stream. This however, I think, is not an insuperable objection. When the bacillus colonizes and multiplies in the joint its effects must be much the same whatever the portal of entry, providing of course that preceding trauma does not play too large a rôle. While trauma is inflicted by injection in experimental work, the puncturing of the capsule by a fine needle is not a great injury. As before stated, contamination by other bacteria did not occur in a single instance of simple injection.

My study adds but little that is definite concerning our knowledge of spontaneous dislocation, as this complication did not occur in any of the experiments. In truth, reasoning from the cases in man, it was not to be expected, since attempts to produce infection of the hip joint were not successful. Generally speaking, the results show how dislocation would be favored by the partial or entire destruction of interarticular ligaments and the joint capsule. In one case, No. 14, these structural changes permitted such excessive motion in the joint that dislocation was not at all a remote possibility.

Finally, the outcome of this study suggests at least two points that might be worthy of consideration in further research upon the subject. First, the employment of larger animals, as hares or even monkeys, in which bacilli could be introduced directly into arteries near the joints. Second, the preparation of typhoid bacilli by growth upon culture media to which was added increasing amounts of the serum of that species of animal to be employed, thus enabling the bacteria to survive for a greater length of time after introduction into those animals.

# DIFFERENTIAL METHODS FOR DETECTING THE TYPHOID BACILLUS IN INFECTED WATER AND MILK.\*

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It is of the greatest importance in tests of the sanitary quality of water and milk supplies that organisms which specifically cause infectious diseases through such sources shall be subject to methods of rapid and definite isolation. Up to the present time this has been a matter of great difficulty and uncertainty owing to the fact that the disease germs which occur in water and especially in milk are intermingled with, and greatly outnumbered by, other intestinal germs and many species of common so-called air and water bacteria.

## CHEMICAL PRECIPITATION AND AGGLUTINATION.

Many methods have been employed in isolating or attempting to isolate the typhoid bacillus from water. Processes involving chemical precipitation have been studied by Vallet,<sup>1</sup> Schüder,<sup>2</sup> Ficker,<sup>3</sup> Müller,<sup>4</sup> Willson,<sup>5</sup> Nietor,<sup>6</sup> and Ditthorn and Gildemeister,<sup>7</sup> the latter using ox bile as an enrichment medium after chemical precipitation.

Agglutination has also been used in enrichment methods for the isolation of *B. typhosus* by Adami and Chopin,<sup>8</sup> Klotz,<sup>9</sup> Shipilewski,<sup>10</sup> and Altschuler.<sup>11</sup>

## INDICATOR DYES AND DIFFERENTIAL CHEMICALS.

Methods have been recommended involving the use of dyes as indicators or of chemicals which favor the growth of typhoid germs and retard or exclude other bacteria. In some cases *B. typhosus* has been isolated by the use of a food medium containing both an indicator dye and a differential chemical.

In 1890 Parietti<sup>12</sup> employed a process consisting of the addition of varying amounts of the water to be tested to a series of broth tubes containing increasing quantities of a solution of 4 per cent hydrochloric acid and 5 per cent of carbolic acid. These he incubated at 37° C. and made cultures from the highest acid concentrations in which

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growth occurred. Some years later, Hankin<sup>13</sup> modified this process by selecting the next highest tube in which growth occurred and by starting a new series of tubes from this growth. The higher inoculations of this second series were then finally examined by subcultures.

Elsner<sup>14</sup> in 1896 recommended a medium having 10 per cent of gelatin and 1 per cent of potassium iodide added to an infusion of potato having a reaction of 3.0 per cent normal acid. By means of Elsner's method or by phenolated gelatin several experimenters,<sup>15 16</sup> were able to isolate the typhoid germ from polluted water.

In 1902, v. Drigalski and Conradi<sup>17</sup> isolated the typhoid bacillus from excreta by nutrose litmus lactose agar containing crystal violet, and Conradi by the use of carbol gelatin plates succeeded in isolating *B. typhosus* from a well at Pecs in Hungary. The Drigalski-Conradi<sup>18</sup> medium is a selective one favoring the growth of *B. typhosus* and *B. coli*. Examinations are made after 24 hours' incubation at 37° C. The colon bacillus produces red non-transparent colonies, while the typhoid colonies are blue or violet and are transparent and of smaller size.

Löffler,<sup>19 20</sup> and also Lentz and Tietz,<sup>21 22</sup> have used malachite green agar as a differential medium. Doebert<sup>23</sup> and Nowack<sup>24</sup> have shown that certain varieties of this dye cannot be used, the latter author attributing the difference to the presence of dextrin.

Endo<sup>25</sup> used fuchsin lactose agar decolorized by sodium sulphite and Gähtgens<sup>26</sup> added to this caffeine which had been found by Roth<sup>27</sup> to inhibit the *B. coli*. On this media the *B. coli* is red and the *B. typhosus* colorless. Hoffman and Ficker<sup>28</sup> in 1904 isolated *B. typhosus* from feces and from infected water by using caffeine-nutrose-crystal-violet solution and transplanting by smears on Drigalski-Conradi plates.

The same year Jaksch and Rau<sup>29</sup> isolated typhoid bacteria from the Prague Water Supply and from the Moldau River, and Ströszner<sup>30</sup> isolated it from a well near Budapest by the use of caffeine-nutrose-crystal-violet agar.

Methods involving chemical precipitation, agglutination, or even the use of stains have a great tendency to reduce the original number of typhoid bacteria present, and in cases where they are at the start very few in numbers there is a danger that they may be lost sight of altogether. This is also true to a greater or lesser extent with acids, caffeine and other chemical substances used for differentiation.

#### DIFFERENTIAL FOOD MEDIA.

A simple and valuable medium proposed by Hiss<sup>31</sup> and used by the authors does not have this disadvantage and after their enrichment is of importance in the differentiation of *B. typhosus* from *B. coli*. Hiss's medium is semifluid at 37° C. and *B. typhosus* has a tendency to swim out from the colonies forming branchlike processes which distinguish it from *B. coli*. In composition this medium is:

Agar .....	15 gm.	NaCl.....	5 gm.
Gelatin .....	15	Dextrose .....	10
Liebig's Extract.....	5	Distilled Water.....	1,000 c.c.
Reaction 1.0 per cent normal acid.			



Other media of this character were originally proposed by Rosenthal<sup>32</sup> in 1895, Klie<sup>33</sup> and Capaldi<sup>34</sup> in 1896, and Hiss in 1897 and 1901. Differentiation with all of these media is based on the high motility of the typhoid bacillus. The differential medium of Hesse which will be described later may be considered to be a refinement of these media which allows of much greater ease in distinguishing between *B. typhosus* and *B. coli*.

#### NEW METHOD.

The method which the authors desire to propose is not subject to the liability of the loss of *B. typhosus* even when present in very small numbers and has proved to be most successful in the numerous cases in which it has been tried. It is based on a preliminary cultivation of the sample of water, milk, or feces to be tested in lactose bile (34, 35, 36) as an enrichment medium and plating from this on Hesse agar (37, 38) to get the characteristic typhoid colonies.

*Examination of typhoid feces.*—Hesse agar may be used directly in the examination of the feces of suspected cases of typhoid fever, but the authors have found in cases where the test was uncertain, due to the outnumbering of the *B. typhosus* by the *B. coli*, that preliminary cultivation in lactose bile insures definite results. The mode of procedure is to plant dilutions of the feces in Hesse agar and at the same time inoculate with the feces a fermentation tube containing lactose bile, both being incubated at 37° C.

If on the second day the cultures from the Hesse agar are uncertain a series of dilutions are made from the lactose-bile tube which, if necessary, may be followed by a third series on the following day from the two-day-old culture in the fermentation tube.

The preliminary cultivation in the lactose-bile solution greatly increases the number of the *B. typhosus* present so as to render its identification more definite. It often gives more characteristic colonies where there is uncertainty in identification and at the same time it preserves the cultures for future transplantations when second or third tests are necessary.

*Lactose-bile medium.*—This medium which was first proposed by one of the authors has now come into general use in testing for *B. coli* in water, and is also used in some laboratories in testing for the amount



and extent of fecal contamination in milk. It consists of sterilized, undiluted fresh ox gall (or an 11 per cent solution of dry fresh ox gall) to which has been added 1 per cent of peptone and 1 per cent of lactose. Fermentation tubes holding 40 c.c. of the sterilized liquid are inoculated with varying amounts up to 10 c.c. of the water or milk to be tested. After 48 hours of incubation at 37° C. at least 25 per cent of the length of the fermentation tube will be filled with gas if *B. coli* is present. In attenuated cultures a three-day test is sometimes required.

The authors have found that *B. typhosus* grows even more abundantly in this media than does *B. coli*, that other organisms are rapidly overgrown, and that *B. coli* itself may be finally overgrown by *B. typhosus*. But as *B. typhosus* does not produce a gas and does not in any other way indicate its presence by the appearance of the medium, it was necessary to go through a very laborious process in order to isolate the organism. While experiments of this character were under consideration, the discovery of a new medium by Dr. W. Hesse was announced.

*Hesse agar*.—This medium easily distinguishes between *B. typhosus* and *B. coli*, and unlike other distinguishing media has no tendency to reduce the number of typhoid bacilli present. By its use the authors were able to isolate *B. typhosus* from preliminary cultivations in lactose bile of samples of suspected water supplies and artificially infected milks. In such cases the preliminary cultivation in bile is necessary in order to increase the typhoid germs in numbers over the various other species always present in contaminated supplies.

The Hesse medium contains less agar and more salt than standard agar and is made from Liebig's extract of beef instead of the pure meat juice. Its composition is as follows:

Agar .....	5 gm. (4½ gm. absolutely dry)
Peptone, Witte.....	10 "
Liebig's Extract of Beef..	5 "
Salt .....	8½ "
Distilled Water.....	1,000 c.c.

In a study of this medium the authors have found:

That the agar must be previously dried for half an hour at 105° C. as it sometimes contains as high as 20 per cent of moisture and

an exact amount of agar is highly important in order that the medium may be very soft but still retain its position on the plate. With agar dried as described the proper amount to use is  $4\frac{1}{2}$  gm. per liter. This is probably equivalent to that used by Hesse, as according to description our agar was more thoroughly dried. A small sample of the agar may be dried and the moisture allowed for in making the medium.

That the medium must be stored in an ice chest the air of which is kept saturated with moisture, and the cultures must be incubated in a  $37^{\circ}$  C. incubator also with moisture-saturated air so that the medium will not change its consistency. It is a decided improvement to use porous earthenware tops<sup>42</sup> to the petri dishes instead of the ordinary glass tops in order to prevent spreading and to keep the colonies in their natural and characteristic condition.

#### PREPARATION OF HESSE AGAR.

The preparation of the medium is in detail as follows: Four and one-half grams of dry agar is dissolved in 500 c.c. of distilled water by heating over a free flame, making up loss in weight by evaporation. Into another vessel 500 c.c. of distilled water is poured and to this is added 10 gm. of peptone, 5 gm. of Liebig's meat extract, and 8.5 gm. of salt. This is heated until all is dissolved and the loss in weight by evaporation is made up by adding distilled water.

Add the two solutions together; boil 30 minutes; make up loss in weight with distilled water, filter through absorbent cotton held in the funnel by cotton flannel, passing the filtrate through several times until perfectly clear. Test the reaction and adjust, if necessary, to 1.0 per cent normal acid, and tube, using 10 c.c. of medium in each tube. Sterilize for twenty minutes at  $120^{\circ}$  C. (15 pounds pressure) in an autoclave. Cool with running tap water and store in an ice chest, the air of which is saturated with moisture.

Extensive experiments have been carried on to see if any improvement could be made in the contents of this medium. Different amounts of agar were tried, from 4 to 7 gm. being used taking each half-gram in between these two points. Standard extract of fresh beef was tried but did not seem to give as good results for the purpose as Liebig's extract. Varying amounts of salt from none to 10 gm. with standard broth and also with Liebig's extract were used but the amount given in the formula was found to give the most characteristic results.

The preparation of dilution plates is carried out as follows:

Eight tubes containing each 9 c.c. of sterilized distilled water are set in a rack together with a series of eight sterilized petri dishes and both numbered from 1 to 8. Dr. Hesse uses physiological-salt solution for dilution but our experiments show that distilled water always gives equally good results.

Into Tube 1 place 1 gm. or 1 c.c. of feces, or 1 c.c. of bile solution containing the feces, water, or milk to be tested and which has been previously inoculated and incubated at least 24 hours at  $37^{\circ}$  C. After thoroughly mixing with the 9 c.c. of distilled

water in the tube place 1 c.c. of this mixture into Plate 1, and 1 c.c. into Tube 2. From Tube 2 place 1 c.c. into Plate 2 and 1 c.c. into Tube 3. Proceed in this manner through the series. Now add to each petri dish 10 c.c. of the liquefied Hesse agar cooled to 40° C. and mix thoroughly. First cool in the ice chest to set the medium and incubator at 37° C. for 24 hours.

The use of physiological-salt solution is unnecessary in the dilution work for the various plates used, but it should be used in the dilution of the anti-typhoid serum, described later. The following table shows that distilled water may be used with equal success in the dilution work previous to plating Hesse agar.

Table 1 shows the use of physiological-salt solution compared with sterilized distilled water for dilution work using three strains of *B. typhosus*:

TABLE 1.

DILUTION	BACTERIA PER C. C.				
	Plate 1 1-10	Plate 2 1-100	Plate 3 1-1,000	Plate 4 1-10,000	Plate 5 1-100,000
Strain No. 1					
NaCl sol.....	1,200	116*	17*	2*	0
Sterile water.....	1,290	97*	15*	2*	0
Strain No. 2					
NaCl sol.....	2,160	227	24*	3*	0
Sterile water.....	2,140	229	24*	2*	0
Strain No. 3					
NaCl sol.....	13,460	1,760	184*	10*	2*
Sterile water.....	13,400	1,740	185*	21*	2*

Plates marked with a star gave very characteristic colonies on Hesse agar but where there was a large number of bacteria present the results were not characteristic.

Strain No. 1 represented an old stock culture of *B. typhosus* from which very characteristic colonies could not be obtained on the medium until after rejuvenation with lactose bile for 12 hours.

Strain No. 2 was a culture obtained from Dr. Billings of the City of N. Y. Board of Health.

Strain No. 3 was a culture from Bellevue Hospital.

When feces are plated directly on Hesse medium, the amount taken and the dilution being known, the number of each characteristic species of bacteria is estimated. When feces are inoculated into bile solution the presence only and not the number of *B. typhosus* is determined.

When milk or water are incubated with bile solution varying dilutions in bile tubes may be made to obtain the approximate number of *B. typhosus* present but in practice the presence of the germ is all that it is necessary to determine.

#### CHARACTERISTICS OF *B. TYPHOSUS*.

*B. typhosus* is characteristic on Hesse agar only when the dilution is sufficiently high to produce but a few bacteria on the plate. It is distinguished from *B. coli* by the formation of colonies of much larger size, often several centimeters in diameter and consisting of a broad translucent or scarcely turbid zone between the white opaque center or nucleus and the perfectly circular narrow white seam or edge. These

characteristics are shown in the accompanying figure giving a dilution of *B. coli* and *B. typhosus* taken by the authors from the Hudson River at Hastings. The culture was previously grown in lactose-bile solution.

The second illustration is a pure culture of *B. typhosus* taken from a stream and pond used as a local private water supply which has been the source of a number of cases of typhoid fever.

These and the other pure cultures of typhoid bacilli isolated were all found to give the proper results when tested by agar and gelatin plates, stabs and slopes, indol reaction, milk, litmus, lactose agar, dextrose broth, lactose bile, potato, Hiss medium, Drigalski-Conradi medium, Hesse agar, morphology, motility, pathogenesis, Pfeiffer reaction, absorption test and Widal reaction <sup>43 44 45 46</sup> (1-1,000). Negative results were obtained on gas, and indol production and positive or characteristic results were obtained by the other tests. Animal subcutaneous inoculations with moderate quantities of 24-hour broth cultures gave negative results. Large quantities produced toxemia.

Additional pure cultures of *B. typhosus* used in the comparative tests were kindly supplied by Dr. Benjamin White of the Hoagland Laboratory, Brooklyn, N. Y., and were from the following sources:

- B. typhosus* K, isolated by Kruse, Bonn, June 1907, from feces.
- B. typhosus* L-1, isolated at Lister Institute, 1907, from feces.
- B. typhosus* L-2, isolated at Lister Institute, 1907, from feces.
- B. typhosus* L-6, isolated at Lister Institute, 1907, from feces.
- B. typhosus* M, isolated by Trommsdorf, Munich, from feces.
- B. typhosus* N, isolated by Neisser, Frankfort, from feces.
- B. typhosus* P, isolated by Besredka, Pasteur Institute, Paris, from feces.
- B. typhosus* H, isolated by Neumann, Heidelberg, June, 1907, from feces.

*B. paratyphosus*, *B. pyocyaneus*, *B. fluorescens liquefaciens*, and some forms of very motile *B. coli* sometimes give a similar appearance. They may be readily distinguished by the absence of a transparent zone and by the Widal reaction. The fermentation test or the planting in the various differential media may be necessary in case of indecision.

The Widal reaction may be made directly from the colonies on the Hesse plate as well as by first planting in beef broth for a period of from 12 to 24 hours.



FIG. 1.—*B. coli* (small colonies) and *B. typhosus* (large colonies) taken from the Hudson River and transplanted from lactose bile to Hesse agar.

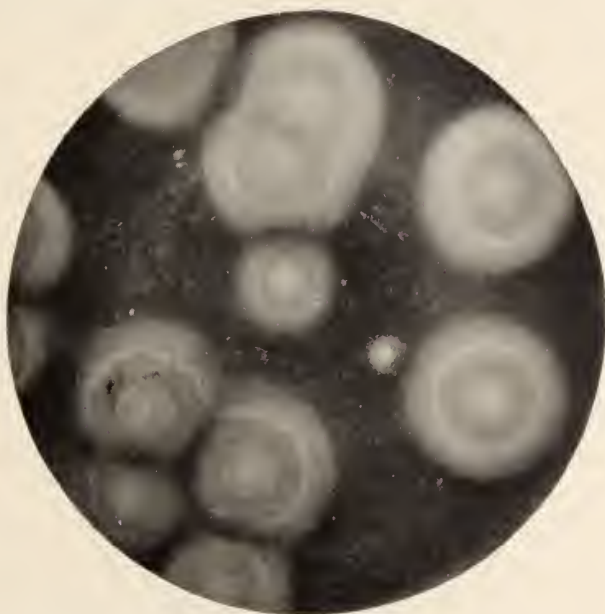


FIG. 2.—Pure culture of *B. typhosus* taken from a stream and pond at Hastings, N. Y., used as a private water supply and the source of several cases of typhoid fever.



## THE WIDAL TEST.

To make the Widal test take up a small portion of the culture in a capillary glass tube, place a drop on a cover glass, and invert it over a rubber ring on a glass slide, using vaseline on the edges so as to prevent evaporation and consequent movement by currents in the drop.

If the bacteria are motile rods resembling *B. typhosus*, add a drop of equal size of highly diluted anti-typhoid serum. If in the course of a few minutes the bacteria cease their motions and agglutinate, the presence of *B. typhosus* is practically established, but it is well to make the other cultural and biochemical tests to see that they correspond to the reactions of the typhoid bacillus. In rare cases some strains of *B. coli* and allied species may give the Widal test, but almost invariably in dilutions of 1-50 or less. The high-power dry lens is best for this work.

To determine the point of highest dilution at which agglutination takes place the Widal test is best made in tubes and examined macroscopically. Place a series of small test tubes in a rack and pour into each varying dilutions of anti-typhoid serum. To each tube add an equal amount of 24-hour broth culture of *B. typhosus* and incubate at 37° C. for at least three hours. The highest point at which precipitation takes place is the highest point of agglutination.

## CONCLUSIONS.

Bile is a natural medium for the growth of the typhoid bacillus and it retards the growth of other bacteria except *B. coli*. When lactose is present in the bile *B. coli* causes copious gas formation and soon produces sufficient acidity to retard its own growth so that the final result is a predominance of *B. typhosus* which has been multiplying rapidly in the meantime and is not materially affected by this acidity.

When samples of water or milk are inoculated into lactose bile and incubated at 37° C. and then transplanted in varying dilutions into Hesse agar, very characteristic colonies are formed in the high dilutions when the typhoid bacillus is present.

By means of the method described we have been able to isolate typhoid cultures from feces with certainty at any stage of the disease. We have isolated it from milk artificially infected with small numbers



of *B. typhosus*. We have isolated it from the Grass River used as a source of water supply for Canton, N. Y., from a pond and stream used as a local private water supply at Hastings, N. Y., and from two points in the Hudson River.

At Canton and Hastings it was obtained from bile tubes containing 10 c.c. of the water, and in the Hudson River near Hastings, at the time of the local typhoid fever epidemic at that point, it was obtained in bile tubes containing only 1 c.c. of the water.

Any volume of water may be tested provided at least four times as much lactose bile as water is added to the bottle before incubation. In routine water analysis when gas is formed in the bile tube, cultures should be plated out on Hesse media to determine the presence of *B. typhosus*. If characteristic colonies are obtained they should be examined for morphology and motility under the microscope and the Widal test applied. This practically demonstrates their presence but they should then be isolated in pure culture and plated on the various distinguishing media as confirmative tests.

The authors desire to express their obligations to H. F. Muer, T. C. Muer, and J. A. Powders, all of Mount Prospect Laboratory, for valuable assistance in the preparation of this paper.

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# PROLIFERATIONS OF THE EPITHELIUM INDUCED BY SUDAN III, SCHARLACH ROTH, AND PARAFFIN; AND THE EFFECTS OF ROENTGEN IRRADIATION UPON THE SAME.\* †

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EVER since the cause of cancer has been a matter of consideration, experimenters have attempted, by various means, to induce cancer in animals, hoping to open the way to ascertain the etiology. Basing their operations, generally, upon some feature in the history presented by persons or animals afflicted with carcinomata, such agencies as direct trauma, heat, electric current, Roentgen irradiation, corrosive chemicals, etc., have been made use of, in efforts to produce a neoplasm of carcinomatous nature. The results of these experiments have been practically uniformly negative.

Based somewhat upon such experimentation, several theories regarding the etiology of cancer have been set forth. These, in their turn, have had the sanction and approval of pathologists in general, only to yield at last under the more diligent and painstaking investigation on the part of their supporters as well as of their opponents.

The outcome of the discussions has brought about a practical discarding of the theory of parasitic relationship to cancer, and a wavering division of pathologists between Ribbert's and Cohnheim's theories, with a few advocates of some minor theories and modifications. No phase of medical research is receiving more attention than the etiology of new growths, and none is more undetermined.

Nearly three years ago Bernard Fischer<sup>1</sup> gave a renewed impetus to experimental investigation into this question by reporting a series of experiments conducted by himself. It is not necessary to review the details of his operations since these are generally familiar. Suffice it to say that, following the subcutaneous injections of certain dyes, in oil, he secured a marked proliferation of epithelium which he considered to resemble the squamous-celled carcinoma in all essentials. He attributed to the dye-oil a property of attracting the epithelium from the surface into the deeper tissues. The active element he named "attraxin," and thereupon he asserted the attraxin theory.

Following Fischer's report a great many laboratory workers set out to duplicate

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<sup>1</sup> *Munch. med. Wchnschr.*, 1906, 53, p. 2041.

and so to confirm his observations, or, failing to secure results similar to his, to challenge the accuracy of his observations and judgment.

A considerable amount of experimentation along this line has been carried out by Snow,<sup>1</sup> McConnell,<sup>2</sup> Helmholtz,<sup>3</sup> and Hertzler.<sup>4</sup> The last three named report work and observations warranting the acceptance of Fischer's findings, as far as the matter of epithelial proliferation is concerned, while Snow's report tended to the opposite conclusion.

Hertzler attributes the results he secured, not to an "attraxin" action on the part



FIG. 1.—Section from ear of rabbit 21 days after injection with soft paraffin. Note the hyperplastic epithelium about the hair follicles. (Snow.)

of the dye and directed toward the cells of the epithelial zone, but to "some disturbance in the chemical relationship of the different kinds of cells." He draws the general conclusion, then, that "when a chemical which has the power of combining with the acidophilic elements is injected into a tissue made up of epithelium and connective tissue, the epithelium proliferates, and invades the connective tissue, simulating the process in beginning epithelioma."

Helmholtz broadened his experiments to the extent of inducing proliferation of epithelium of mucous membranes, as well as of the skin. In one of his specimens he has demonstrated a multiple chondroma which he considers as being a consequence of the action of the injected dye.

<sup>1</sup> *Jour. Infect. Dis.*, 1907, 4, p. 385.

<sup>3</sup> *Johns Hopkins Hosp. Bull.*, 1908, 18, pp. 365, 369.

<sup>2</sup> *Jour. Amer. Med. Assoc.*, 1907, 49, p. 1498. <sup>4</sup> *Jour. Amer. Med. Assoc.*, 1908, 50, p. 425.

The work in this laboratory has been limited to that done by Snow. His first set of experiments was reported in June, 1907, and in so far as agreeing with Fischer's results they were decidedly negative. Later, in work not reported, he secured a hyperplasia of epithelium under the influence of Sudan III in olive oil. In addition to this he secured a marked hyperplasia of epithelium from injections of soft paraffin in paraffin oil. He has kindly placed the results of this work at my disposal with permission to report the same herewith (Fig. 1).

From the many sets of experiments, then, it can be asserted that injection of certain dyes, as Sudan III and Scharlach Roth, in olive oil does cause a proliferation of the epithelial cells. Practical application of this fact is being made in the use of ointments containing a small percentage of these dyes, to stimulate the processes in skin grafting. Schmieden<sup>1</sup> reports very successful work in this line from an 8 per cent salve of Scharlach Roth. He considers the process safe, without any tendency to cancer formation, and attributes to the dye a specific chemotactic action toward the epithelial cells.<sup>2</sup>

Basing our judgments upon the work done in this laboratory, it has been maintained from the first, by the experimenters and observers here, that this produced condition does not correspond in essential details with the squamous-cell carcinoma.

At the suggestion of Dr. Warthin, and under his directions, I have undertaken to repeat Snow's experiments and, in addition, to ascertain the effects of Roentgen irradiation on this induced epithelial proliferation.<sup>3</sup>

Inasmuch as only the general effect of the X-ray was sought, i. e., whether the irradiation tends to stimulate or to inhibit the process, many of the minor details, necessary to be considered in a complete study of the question, were omitted. No exact measurements of the irradiations were made. In all cases medium tubes were used, at eight inches distance from the parts exposed, and with ten minutes' exposures. The solution injected was a saturated solution of Sudan III in pure olive oil. This solution, having been proven to have no antiseptic property in itself (report from the Department of Bacteriology), was rendered sterile by fractional sterilization, and was always injected with care regarding infection.

The injections were made as superficially as possible in the lateral external surface of rabbits' ears. The precaution to make the injections as superficially as possible was used at the suggestion of Hertz-

<sup>1</sup> *Centralbl. f. Chir.*, 1908, 6, p. 153.

<sup>2</sup> Carnezzì (*Gaz. degli Ospedali e delle Cliniche*, Milan, 1900, February 2, No. 14) reports very satisfactory results along the lines of Schmieden's work using 8-10 per cent Scharlach Roth salve to promote epithelial growth in the process of skin grafting.

<sup>3</sup> My obligations are due to Professor Warthin for the original suggestion to do this piece of work, and for further instructions as the work progressed, also for assistance in securing the literature bearing upon the subject. I am indebted also to Mr. V. J. Willey of the University Laboratory of Roentgenology for assistance in his laboratory, and for permission to use his apparatus for the irradiations.



ler, who further says that Snow's failure to secure satisfactory results from his first work may be attributed to the injections being made too deep into the tissue.

At first a few ears were used to determine the separate effects of X-ray and of Sudan III.

EXPERIMENT 1: *To ascertain the effects of the X-ray alone.*—Two Belgian hares were used for this purpose. Throughout a period of 14 days ears were exposed under the conditions stated above. One ear was irradiated every second day, two daily, and one twice a day. In each case the animals were so protected by lead plates as to restrict the irradiation to the ear desired to be exposed.

The results of these experiments are simple. The effects upon the epithelium, with which alone in all of these experiments we are concerned, consist simply of an increased pigmentation of the basal cells. This pigmentation is least marked in the ear exposed every second day, and most marked in the ear exposed twice a day. The amount of pigment, however, does not seem to be dependent absolutely upon the amount of irradiation, as some ears show more increase in pigment under daily exposures than some others do from exposures twice a day. No special pains were taken to account for these differences, but it was thought that this is, perhaps, dependent upon the specific amount of pigment normally present in the skin of a given animal. The black rabbits showed a greater increase in pigmentation than the white or gray ones did. The amount of increase in pigmentation was judged by comparing sections from the irradiated ear with sections taken before the irradiation or with sections from the base of the ears which were protected by the lead plates. Beyond this pigmentation there was no change considerable with the question at hand. There was certainly no sign of hyperplasia, but rather an atrophy of the elements of the tissue.

EXPERIMENT 2: *To ascertain the effects of Sudan III-oil alone, also under the influence of Roentgen irradiation.*—Five Belgian hares were used for this purpose. Both ears of each animal were injected with Sudan III-oil. The amounts injected, the sites of the injections, and all conditions were identical in both ears of each animal. One week later the injections were repeated with the same precautions. At the end of the second week pieces were removed from all ears, fixed in formalin and in Zenker's solution, and stained in hematoxylin and eosin. During the course of the two weeks the several ears were given Roentgen irradiation at intervals of from twice a day to once every second day. In every instance the two ears of each animal were given different amounts of irradiation for sake of comparison, which was considered preferable to comparing the ears of different rabbits in any case.



The detailed findings in but three of the animals will be given here, as the other two served only as duplicates and gave the same results.

*Hare 1.*—Both ears were injected, according to the conditions outlined above. The left ear was not irradiated. The right ear was irradiated once every second day for two weeks.

But slight differences could be noticed between the gross appearances of the two ears during the time of treatment. Both ears thickened somewhat and presented a scaly surface. The right ear was somewhat darker than the left, but not enough to be considered at all marked.

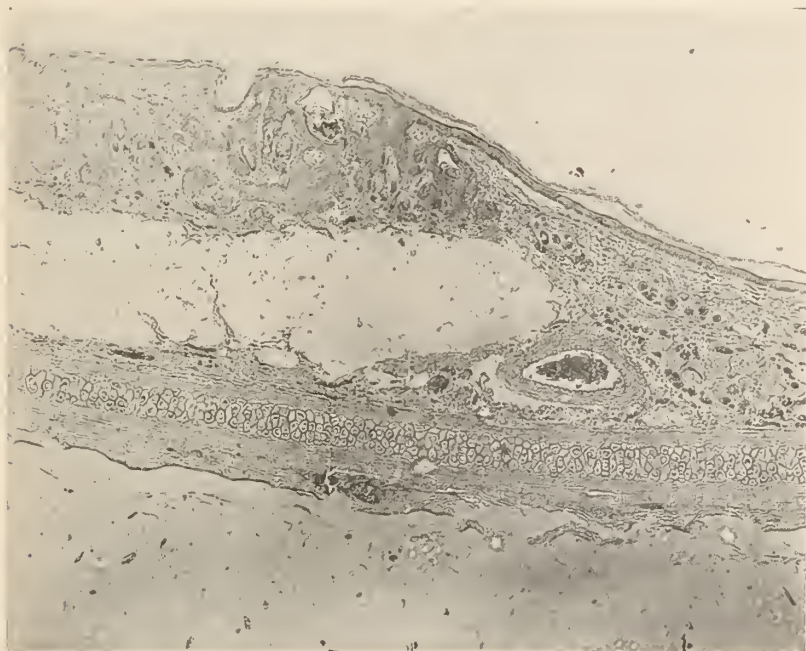


FIG. 2.—Section from ear of rabbit 14 days after injection with a saturated solution of Sudan III in olive oil. Note the extensive proliferation of epithelium showing arrangements resembling epithelial pearls and ingrowths from the surface layer of cells. These are, in fact, the masses of hyperplastic epithelium about hair follicles, which have been cut at various angles and so have this carcinomatous appearance. The portion of the figure at the right side shows the normal epithelium and hair follicles.

In this case, and to a greater degree in the next two cases, there was noted a difference in temperature in the two ears—the irradiated ear in each case feeling decidedly cool as compared with the mate.

On section these show practically the same features which have been described by several observers (Figs. 2 and 3). The most marked change from the normal picture is shown in the amount and the arrangement of the epithelium. The marginal layer of cells is increased in thickness, the cells staining practically the same as in the

normal condition. The great change in appearance is about the hair follicles. Here there is an extensive hyperplasia of epithelium, giving rise to large, some oval, some irregularly shaped, patches of cells. Most of these areas distinctly show themselves to be associated with hairs, while others, if considered in and of themselves, would give no suggestion of relation to hair follicles. It is easy to find areas, circular in outline, in which the concentric arrangement of the cells is very evident, the inner rows taking the stain less distinctly, and in all respects resembling the epithelium pearls of the squamous-

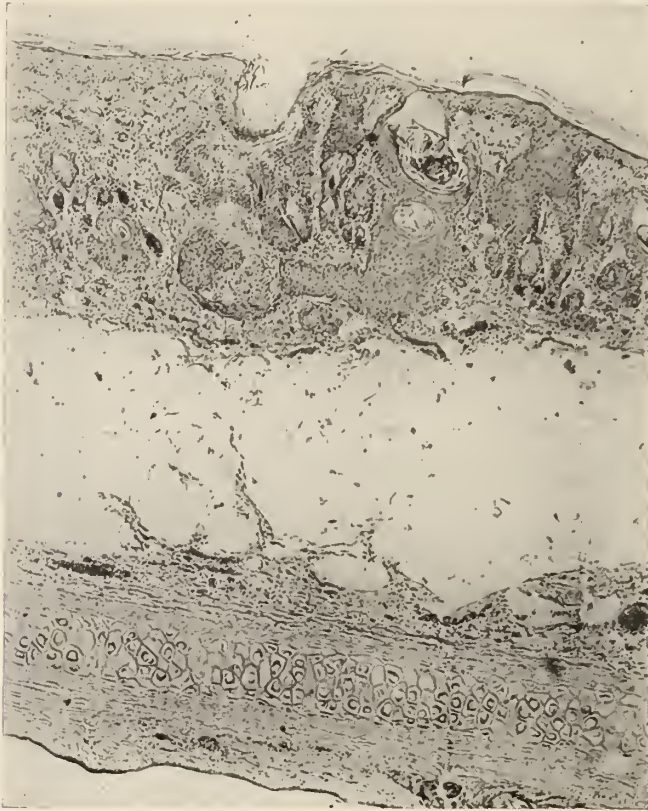


FIG. 3.—Higher magnification of section shown in Fig. 2.

cell carcinoma. Then, too, it is possible to find places where the mass of epithelial cells deep in the tissues is so connected with the surface as to appear to be a direct ingrowth of the surface zone of cells. Both of these features, which have been described as marking a close relationship between this process and that of carcinoma, can easily be accounted for as coming in all cases from the proliferation of cells about the hair follicles, if we consider the possibilities there are in this direction resulting from the cutting of sections through these areas. Serial sections easily prove the truth of this point.

This point was insisted upon by SNOW, in his report cited above, as explaining the striking features of the sections offered by some workers at that time. These, as well as the cysts described, I believe are all derived from the hyperplasia of the epithelium about hair follicles.

At no place have I seen the epithelium invading the connective tissue or the cartilage, but there are places where the cartilage plate is the limit of the growth, and should such a proliferation occur immediately over one of the normal interruptions of the cartilage plate, it might extend between the two portions, and so appear to have grown into, or even entirely through, the cartilage.

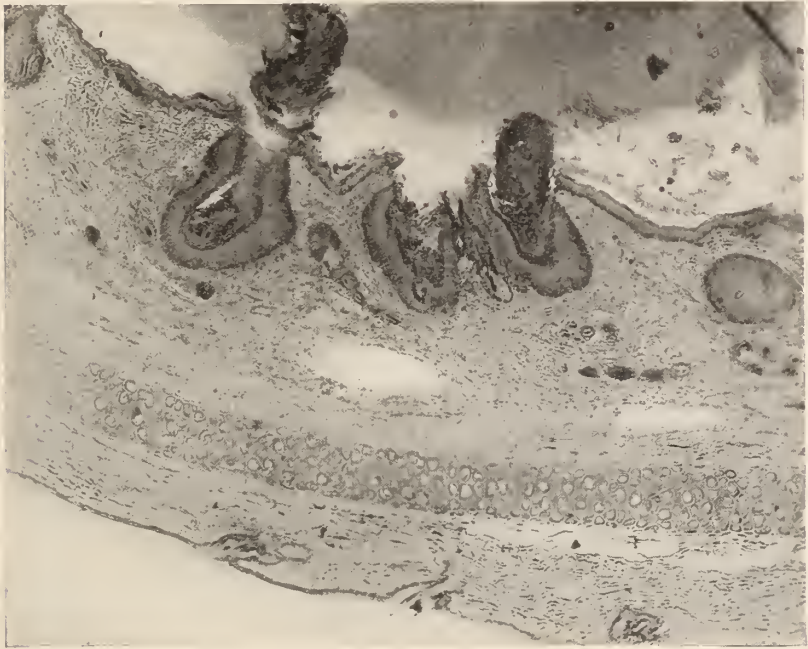


FIG. 4.—Section from ear of rabbit 14 days after injection with a saturated solution of Sudan III in olive oil, during which time the ear was irradiated daily. Note the lessened amount of epithelial hyperplasia which is localized about the hair follicles. Also note the pigmentation of the basal cells of the surface zone and the peripheral cells of the follicular masses.

The clear, generally oval, sometimes irregularly shaped spaces are oil spaces. These have no wall except the connective tissue, which is somewhat compressed and the inner layer flattened, so that it has in some places the appearance of an endothelium or of a flattened epithelium. I find no place, however, where it would be safe to say that epithelial proliferation has taken place about one of these oil spaces. These are the conditions in the left ear, which was not irradiated.

In the right ear, irradiated every second day, these same features exist, and are different from those in the left only in degree. The examination of a single picked section from each ear might leave one in doubt as to any existing difference, but after

running over a great many sections from each ear, the conclusion that there is less of cell proliferation in the irradiated ear is easily arrived at. The cells are less distinctly stained in these sections, especially about the hair follicles, than in the set just described. The irradiated ear shows the same increased pigmentation as that seen in the irradiated non-injected rabbits' ears.

*Hare 2.*—Both ears were injected under conditions stated above. The right ear was exposed to the X-rays daily, while the left ear was irradiated only every second day for two weeks. Sets of sections were made from the ears, as in the preceding case.

Here we have all the features given in the sections from the first animal, with,



FIG. 5.—Section from ear of rabbit 14 days after injection with a saturated solution of Sudan III in olive oil, during which time the ear was irradiated twice each day. Note the absence of epithelial proliferation. Also note the intense pigmentation.

however, a more marked diminution in epithelial growth (Fig. 4). There are patches about the hair follicles, as before, but it does not give one the impression of having developed so abundantly as in the other cases. The line of pigmentation is distinct in the sections from the ear that was irradiated daily, and is continuous around all the projections and isolated areas of epithelium. This distribution of pigment does not agree with that seen in epitheliomata after treatment with X-rays, inasmuch as in irradiated carcinomata of the skin pigment is formed only in the rete of normal skin. The series of sections show that there is less proliferation in the right ear than in the left.

*Hare 3.*—Both ears were injected as in previous cases. The right ear was exposed



to the X-rays daily, and the left ear was irradiated twice each day for two weeks. The sections in this case show much less of proliferation in both ears. In the right ear, irradiated daily, there are thickenings about the hair follicles, but not extending deep into the tissues, and showing but few and small isolated areas. These are everywhere bounded by the heavily pigmented layer of cells. In the left ear, irradiated twice a day, the amount and distribution of epithelium differ but little from that in the normal, untreated ear (Fig. 5). There are a few slight thickenings about the hair follicles, but no outgrowths or isolated patches. The basement layer of cells is very heavily pigmented. The oil spaces are very numerous in these sections, showing that the inactivity is not due to a lack of Sudan III-oil.

#### CONCLUSIONS FROM THE FOREGOING EXPERIMENTS.

1. Sudan III-oil may induce a proliferation of epithelium. This proliferation in no sense simulates carcinoma, but is merely a simple hyperplasia of the epithelium about the hair follicles, and in single sections may be mistaken for an infiltration. There is, however, no true infiltration of connective tissue or of cartilage; the normal relationships and characteristics of the cell-layers of the epidermis are preserved; and the hyperplasia can in no sense be regarded as analogous to a neoplastic epithelial proliferation.

2. Whether this hyperplasia is due to the Sudan III or to the olive oil is a question. Snow's securing a hyperplasia from the use of paraffin oil alone makes this an open question, and does not bear out Hertzler's explanation of the proliferation as being due to the removal of the restraining influence upon the epithelium by the combination of the injected material with the connective tissue.

3. Roentgen irradiation inhibits this proliferation, and the irradiation is more efficient when frequently applied.

This last conclusion is in accord with what one would expect after reading the literature bearing upon the effects of Roentgen irradiation upon the tissues, especially upon the elements of the skin. The complete literature upon this subject has been assembled by Professor Warthin.<sup>1</sup> It is not thought necessary to reproduce at all extensively that report here. In practically every case in which an active process was under consideration, it was shown that the X-rays have a restraining influence. As somewhat closely related to this set of experiments, the work of Oudin, Barthélemy, and Darier<sup>2</sup> may be cited.

<sup>1</sup> *Internat. Clin.*, 1906, 15, s. iv, p. 243; *Physician and Surgeon*, 1907, 29, p. 1.

<sup>2</sup> *Monatshfte f. prakt. Dermatol.*, 25, p. 417.

These experimenters secured in guinea-pigs a marked atrophy of the hair follicles from exposure to X-rays. Later, Jutassy<sup>1</sup> secured the same results in irradiated rabbits. The use of conclusions from such experiments as these to strengthen the policy of using X-ray in the treatment of epitheliomata is more or less justifiable, inasmuch as in both conditions the prominent feature is a proliferation of epithelium. Perhaps by continued work along this line, which affords an excellent means for control experimentation, the most efficient strengths and



FIG. 6.—Section from ear of rabbit 14 days after injection with paraffin oil. Note the inflammatory reaction and the hyperplastic hair follicles.

conditions of irradiation can be more accurately determined and so better results secured.

Two additional experiments were suggested by the foregoing.

EXPERIMENT 3: *An attempt to reproduce Snow's results of hyperplasia under the influence of paraffin oil alone, also of olive oil alone.*—Three Belgian hares were used for this purpose. One ear of each animal was injected with paraffin oil, and the other ear injected with olive oil. Within a few days the ears thickened and scaled somewhat.

<sup>1</sup> *Fortschr. a. d. Gebiete der Roentgenstrahlen*, 1899.



The injections were repeated at the end of a week. At the end of the second week pieces were removed from all ears, and examined in the usual way. All of the specimens show a rather marked reaction toward the foreign substances. The vessels show congestion, and within the tissues are many phagocytes. In the ears injected with paraffin oil there is shown a hyperplasia of epithelium about the hair follicles, as compared with other ears of the same animals (Fig. 6). No hyperplasia was secured in the tissues injected with olive oil.

This experiment tends to show that the condition produced by use of the Sudan III-oil was due to the Sudan III, or possibly to the

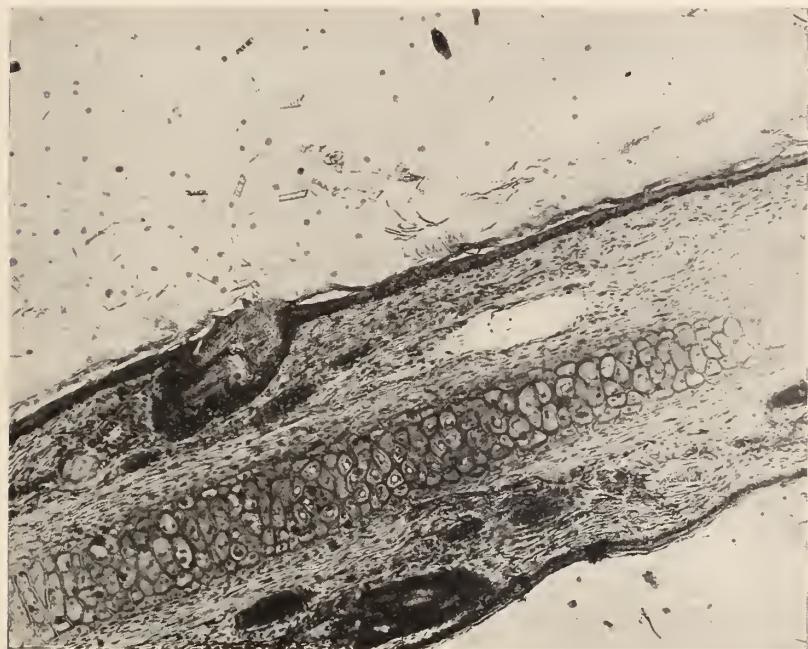


FIG. 7.—Section from ear of rabbit which was irradiated twice a day for 14 days and then injected with a saturated solution of Sudan III in olive oil. The section represents the condition 14 days after the injection. Note the resemblance of this section to the one shown in Fig. 5. Note the intense pigmentation and lack of epithelial hyperplasia.

combination of dye and oil, but not due to the olive oil alone. This may be explained by the more irritant property of the Sudan III and of the paraffin oil, as compared with the bland olive oil. It also shows that this condition does not depend upon the presence of a substance which combines with the tissue elements. And, furthermore, it serves to establish the work done by Snow in this laboratory.

EXPERIMENT 4: *To ascertain the effects of Sudan III-oil after Roentgen irradiation.*—Two Belgian hares were used. One ear of each animal was irradiated daily, and the other ear of each twice a day (two days only once) for two weeks. Then all the ears were injected with Sudan III-oil as in the previous cases, and the irradiations suspended. The tissues (Fig. 7), at the end of two weeks, showed the pigmentation characteristic of the X-ray effect, the oil spaces, some inflammatory reaction, but nothing that could be construed as an increased growth of the epithelium.

This experiment gives us further evidence of the restraining influence of the Roentgen rays, also the persistence of this inhibitory effect after the suspension of the exposures.

NOTE.—Since this work was reported at the meeting of the American Association of Pathologists and Bacteriologists, April, 1908, Werner<sup>1</sup> has conducted a set of experiments with reference to the effects of Scharlach Roth injections into mouse tumors. He arrives at the same conclusion, i. e., that this proliferation is simply the result of irritation and not due to any "attraxin" principle on the part of the dye. This we consider as a confirmation of the conclusion arrived at in this laboratory by the work of Snow and myself, that all of the proliferation obtained from injections of Sudan III, Scharlach Roth, paraffin, and paraffin-oil is purely an irritation hyperplasia having no neoplastic characteristics.

<sup>1</sup> *Munch. med. Wchnschr.*, 1908, 55, p. 2265.

## AUTOLYSIS OF THE GONOCOCCUS.\*

C. T. McCLINTOCK AND L. T. CLARK.

(From Research Laboratories of Parke, Davis and Co., Detroit, Mich.)

IN 1899 Emmerich and Low<sup>1</sup> reported results of extensive experiments in which they called attention to the presence and action of a ferment in *B. pyocyaneus*. According to their reports, they were able to recover this ferment in appreciable amount and with this material in a concentrated form secured results which indicated that the ferment was destructive to a number of species of micro-organisms, among which was included the gonococcus.

Flexner<sup>2</sup> demonstrated that the vitality of the meningococcus was very materially lessened and the germs themselves partially dissolved when they were allowed to remain in suspension in salt solution. He found the products of the disintegration to be detrimental to a luxuriant development of that organism on the medium upon which they had grown and on a fresh medium to which some of the products of disintegration had been transferred.

A biological comparison of the gonococcus and *M. intracellularis* by Martha Wollstein<sup>3</sup> substantiates Flexner's earlier work and indicates that a ferment of decided activity is present in or about the micrococcus of gonorrhea. In producing an antigen Torrey<sup>4</sup> suspended a large and luxuriant growth of the gonococcus (in salt solution) and after submitting this suspension to a constant agitation for a number of hours, he filtered. The resulting clear filtrate was decidedly toxic.

The work of Wollstein, Torrey, and others indicates that a cell destruction occurs in cultures of the gonococcus when placed under certain conditions, the most important of which are temperature, concentration of medium, and age of culture.

With the supposition well established that autolysis of the gonococcus occurs under certain conditions it was the object of our work: (1) To test the results obtained by others; (2) to measure the extent of autolysis and amount of autolytic ferment present; (3) to determine the effect of autolysate on the growth of the gonococcus

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<sup>1</sup> *Ztschr. f. Hyg.*, 1899, 31, p. 1.

<sup>3</sup> *Ibid.*, 1907, 9 p. 588.

<sup>2</sup> *Jour. Exper. Med.*, 1907, 9, p. 105.

<sup>4</sup> *Jour. Med. Res.*, 1907, 16, p. 320.

and other organisms in vitro; and (4) from results of the first three to note the effect of a concentrated autolysate on the organism in vivo and its possible therapeutic application.

When the isolation and cultivation of the gonococcus was first undertaken by one of us (Clark), it was observed that considerable variation occurred in the depth of stain taken by the cocci and later that a suspension of the gonococcus in salt solution became appreciably less dense when left at room temperature for 12 to 24 hours. The loss in density was at first attributed to sedimentation. The changed character of the sediment especially noticeable in its tenacity and the observation of numerous fragmentary cell outlines under the microscope led us to conclude that the cocci had disintegrated at a rate far greater than was to be expected in an isotonic solution.

To obtain a point in departure from which loss in staining qualities, supposedly due directly or indirectly to changes in cell content, and the possible disintegration of the cells could be measured, an attempt was made to prepare a suspension of the gonococcus which would show none of these changes, the lack of such changes being determined by the unaltered appearance of the stained germs under the microscope and the maintenance of a constant bacterial count.

*Staining qualities (qualitative tests).*—A heavy suspension was secured from 20-hour cultures of the gonococcus on ascitic agar. This suspension was divided into two equal portions; to one was added 4 per cent trikresol and then both were sealed up in 1 c.c. amber glass bulbs. These lots were again subdivided into portions; one portion each being set aside without heating it and the remaining portions being subjected to varying degrees of heat for one hour and kept in the dark at room temperature. At the end of one and five days respectively a sample bulb from each lot was well shaken, opened, and a loopful of the suspension stained with methylene blue.

TABLE 1.

STAIN MADE	WITHOUT TRIKRESOL				4 PER CENT TRIKRESOL			
	No Heat	50° C. 1 hour	60° C. 1 hour	70° C. 1 hour	No Heat	50° C. 1 hour	60° C. 1 hour	70° C. 1 hour
After 24 hrs.	none	very few	none	numerous	none	very few	numerous	numerous
5 days	"	none	very few	"	"	none	"	"

Suspension contained 3,388,000,000 germs per c.c.

The results show:

1. Gonococci in suspension receiving neither heat nor trikresol lose completely their original staining qualities.

2. Trikresol alone preserves the staining qualities to a limited extent only.

3. In the presence of trikresol, the preservation of the cells is proportional to the amount of heat to which they are subjected; 60° C. and above for one hour causes them to retain their staining qualities almost perfectly.

4. In the absence of trikresol a greater degree of heat is required to accomplish the same result, 60° C. for one hour with trikresol being equivalent to 70° C. for one hour in the absence of trikresol.

We thus found that a permanent suspension so far as staining qualities are concerned can be secured by submitting the original suspension to 60° C. for one hour in the presence of trikresol or 70° C. for one hour in the absence of trikresol.

*Bacterial counts (quantitative).*—A heavy suspension of the gonococcus was prepared and immediately counted. It was allowed to remain at room temperature and counted at intervals during the succeeding 24 hours. Technique employed was similar to that described by Miller<sup>1</sup> in standardizing bacterial vaccines. Fresh blood from a small puncture in the finger is drawn into a blood-counting pipette to the first small division; a bubble of air is then admitted and one division of the suspension to be counted is taken in; the two equal portions are then thoroughly mixed on a clean slide; and finally small drops are placed on clean slides and spread as for making a differential blood count. Slides are then stained two minutes with Hastings' stain and the bacteria and red blood cells in a given number of fields counted; the number of germs per c.c. is concluded from these results basing the known factor on the number of red blood cells found per c.c. of blood used in making the count.

From a number of tests made the following is given as typical of results obtained:

TABLE 2.

	TIME OF COUNTS					
	9:00 A. M.	9:30 A. M.	10:00 A. M.	2:00 P. M.	5:00 P. M.	8:30 A. M. (23½ hrs. later)
Result of Counts . .	336,575,000	heated 50° C. ½ hr.	244,540,000	139,000,000	135,540,000	113,000,000
Result of Counts . .	180,000,000	heated 70° C. ½ hr.	83,000,000	104,000,000	83,000,000	89,000,000

These results are summarized as follows:

1. In one-half hour at room temperature before heating approximately 28 per cent of the cocci lost the power to take the stain.

2. During the following 22½ hours after heating at 50° C. for 30 minutes, a further 38 per cent of its original number lost their staining

<sup>1</sup> *Ther. Gaz.*, 1907, 23, p. 173.



power, making a total loss of 65 per cent; the decrease, however, being most rapid in the first five hours.

3. A gradual decrease in the number of staining organisms is observed after submitting the suspension to 50° C. for 30 minutes.

It is clearly shown from the table and summaries that a temperature of 50° C. for one-half hour does not prevent disintegration. Subsequent to this test, a suspension was counted, subjected to a temperature of 70° C. for one-half hour, and recounted at intervals. The suspension employed again showed a decided loss during the interval between taking up and heating. After heating at 70° C. for one-half hour no decrease in the number of stained organisms was observed, counts revealing the same number after one and 18 hours respectively.

These results confirm the simple staining results by showing that 70° C. for one-half hour prevents all disintegration and produces a permanent suspension and adds to these results the rate at which disintegration occurs.

Having thus demonstrated to our entire satisfaction that a change occurs in the cell which robs it of its original characteristics, questions presented themselves: (a) Is the loss of staining qualities due to a diffusion of contents through the cell wall? (b) Is the change within the cell wall itself? or (c) Does a break in the cell wall allow the contents to escape into the surrounding liquid?

We found that a thoroughly washed, 24-hour culture of the gonococcus after standing in salt solution for a time liberated sufficient quantities of proteid to give a precipitate with dilute acetic acid and heat. Making use of this fact as a basis for future work considerable progress was made toward a solution of the problems in question.

Large amounts of the gonococcus were grown on ascitic agar and taken up in a small quantity of salt solution. A perfect suspension was produced by shaking, the germs being thrown down by centrifugalizing, the supernatant liquid removed, and the germ residue again suspended in salt solution. This process was repeated until the Berkefeld filtrate from the supernatant liquid was free from proteid as shown by failure to precipitate with acetic acid and heat. After making sure by this method that all traces of the culture medium and soluble proteid were removed, the suspension of germs was divided into portions and allowed to stand at different temperatures. After 6, 18, and 24 hours, the suspensions were again centrifugalized and the supernatant liquid filtered and tested for proteid.



The technique described was repeated on several lots of suspension with decidedly uniform results. The portion kept at room temperature for lengths of time varying from 6 to 24 hours gave a heavy precipitate with dilute acetic acid and heat. The portion heated at 70° C. for one-half hour did not give a precipitation with acetic acid and heat, which fact alone substantiates earlier findings.

These results are in themselves conclusive. Products of change can be detected in the suspending fluid by chemical means. Just what is the nature of this change is not, however, proven by this experiment. It may be partial disintegration of cell wall or diffusion of cell contents through cell wall. From frequent microscopical studies of the partially autolysed suspensions, we are led to favor the first assumption. Numerous irregular, imperfect outlines of the coccus are found to have taken the stain normally, while the ordinarily readily stained interior remains unchanged; consequently, we conclude that a partial disintegration of the cell wall sufficient to liberate contents occurs, although not extensive enough to destroy the staining properties of the wall.

#### ACTION OF ALCOHOL ON AUTOLYSIS.

Those phases of the work dealing with the effect of heat and preservative on the staining qualities of the gonococcus reveal the fact that heat at 70° C. preserves staining qualities, that is, prevents disintegration. The effect of alcohol in varying dilutions on suspensions of the gonococcus was tested as follows:

Four drops of a fresh suspension were placed in tubes of normal solution containing 2 c.c. of 95 per cent, 50 per cent, 25 per cent, 10 per cent, 5 per cent, and no alcohol respectively. Every two hours one loopful of suspension from each tube was stained two minutes with Löffler's methylene blue and a comparison made of slides thus obtained during 24 hours. At the time of taking suspension for smears, a comparative record of its density was also noted. In tubes containing 95 per cent and 50 per cent alcohol, the bacteria rapidly settled to the bottom owing to the low specific gravity of the fluid, but slight agitation restored the suspension, showing that true proteid precipitation had not occurred.

The results are shown in Table 3.

Briefly, alcohol in 50 per cent and 95 per cent dilutions preserves the staining qualities of the germs. In lower dilutions changes occurred, as is shown by loss in density and by imperfect staining in all tubes except those containing the two highest percentages of alcohol.

At this stage of our investigations, after we had proven that a rapid change occurs in suspension of the gonococcus (autolysis), when left under ordinary room conditions, and that such changes can be controlled and prevented at will by the use of heat, trikresol, and alcohol, it became desirable to determine the effect of such disintegration products upon fresh, active cultures of the gonococcus on fresh culture media.

TABLE 3.

SUSPENDING FLUID		TIME OF EXAMINATIONS				
		9:30 A. M.	11:15 A. M.	1:15 P. M.	3:15 P. M.	10:00 A. M. (24½ hrs. later)
Alcohol 95 per cent	Sediment Precipitations Stain	heavy none deeply	heavy slight deeply	heavy slight deeply	heavy slight deeply	heavy slight deeply
Alcohol 50 per cent in NaCl sol.	Sediment Precipitations Stain	heavy none deeply	heavy slight deeply	heavy slight deeply	heavy slight deeply	heavy slight deeply
Alcohol 25 per cent in NaCl sol.	Sediment Precipitations Stain	medium none deeply	heavy none medium	slight none medium	none none o	slight none faint
Alcohol 10 per cent in NaCl sol.	Sediment Precipitations Stain	slight none medium	heavy none deeply	slight none medium	slight o o	slight none very faint
Alcohol 5 per cent in NaCl sol.	Sediment Precipitations Stain	none none medium	slight none medium	none none medium	slight none very faint	none none very faint
NaCl sol.	Sediment Precipitations Stain	none none deeply	none none medium	none none faint	none none faint	none none none

An autolysate was prepared by macerating a large amount of growth of the gonococcus in a small amount of salt solution. The bottle containing this heavy suspension and a number of small glass beads was placed in a mechanical shaker and shaken continually for six hours. Tests on the autolysate proved it to be sterile after 24 hours. A few drops of this sterile material were flowed over the surface of inclined ascitic agar, Thalmann's agar, and plain agar. In 48 hours at incubator temperature the autolysate had become entirely absorbed by the agar, at which time the tubes, together with the control tubes, were planted with equal quantities of a gonococcus suspension taken directly from a luxuriant, viable, 24-hour culture. All tubes were incubated for 48 hours and the effect of the autolysate on the growth of the germs noted.

This experiment was repeated several times and from results obtained we are able to make the following statements.:

1. The autolysate on ascitic agar lessened the amount of growth by more than one-half over that obtained on untreated media.

2. When added to Thalmann's agar, the autolysate had the effect of completely inhibiting the growth.

3. In one set of tests, the gonococcus grew on plain agar alone, while the presence of the autolysate completely prevented development.

#### CONCLUSIONS.

Disintegration or autolysis occurs in gonococcus cultures to a marked degree.

This autolysis can be completely prevented by heat alone at 70° C. for one hour, by heat in the presence of trikresol at 60° C. for one hour, by 50 per cent and 95 per cent alcohol in salt solution, and partially by 0.4 per cent trikresol alone.

Specimens stained at certain stages of disintegration show numerous fragmentary cell walls still capable of taking the stain, indicating that the cell wall is not completely disintegrated.

The presence of shadow forms in which the cell wall only is stained indicates that the cell contents are either so changed as to be incapable of taking the stain or have escaped from within the wall.

The presence of soluble proteid substances (as shown by their precipitation with acetic acid) in the surrounding liquid indicates that the contents escape from within the cell.

Hence, it would seem that autolysis of the gonococcus is effected by rupture of the cell wall and escape of the contents.

The products of this autolytic process markedly inhibit the growth of the gonococcus on artificial culture media. Their use in combating the disease in man will form the subject of a future communication.

# IMMUNE BODIES IN URINARY INFECTIONS WITH COLON BACILLI AND TREATMENT BY INOCULATION.\*

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STUDIES on infections of the urinary tract with especial reference to bacilli of the colon group have been made by a number of observers, notably by Rovsing, Hallé, Krogius, Brown, Dudgeon, and Wilson.

General conclusions reached by the writers are: first, the colon bacillus is the most common infection of the urinary tract; second, these infections may be acute, or chronic, the latter sometimes lasting for years; third, the most common causes are obstruction to the outflow of urine, pressure upon some part of the urinary tract or organs, and invasion of these structures from the intestinal tract, from the exterior through the urethra, or from the blood; fourth, it is common to find strains of colon bacilli in these cases differing markedly in their biological reactions; fifth, agglutination is rarely obtained even in low dilutions (Dudgeon) and is of little or no value for diagnosis.

Among others, Dudgeon<sup>1</sup> has studied cases of this kind with respect to the opsonic content of the serum and the treatment with killed bacteria. He found as a rule a low opsonic index, which by proper inoculation could be raised, and in some cases this rise was accompanied by improvement in the condition of the patient. In some cases, too, the urine improved greatly though this did not always correspond to the clinical improvement. Relapses were liable to occur. He concludes that the results justify the use of the autogenous bacillus.

Dudgeon did not find any appreciable bactericidal action of either normal or patient's serum upon strains of colon bacilli isolated from the urine. Neither did he find any increase in the bactericidal power of anticolon horse serum.

The results reported in this paper deal chiefly with the properties of serum toward the infecting organism, and with the inoculation

\* Received for publication February 19, 1909.

<sup>1</sup> *Lancet*, 1908, 86, p. 615.

treatment. The study centers round certain of the cases whose sera presented unusual features with respect to their bactericidal action. The possible identity of the immune bodies concerned in phagocytosis and bacteriolysis, and the reciprocal adaptation of bacteria and host, are also discussed.

In eight cases of urinary infection the infecting bacilli have been isolated, and the cultural properties studied as well as the effect of normal and homologous serum upon the organism in question. The bacteria vary much both in morphology and cultural reactions. Some are short and plump; others slender and long; some grow profusely on ordinary media, others give a scant growth; all grow under anaerobic and aerobic conditions; two strains give a more profuse growth anaerobically than aerobically. Some coagulate milk, others do not; all acidify it. They vary much in the rapidity with which they ferment sugars. All produce indol. Two strains are hemolytic, producing clear zones on blood-agar plates; the others are not hemolytic.

The important features of the cases are here given.

The first case studied is of unusual interest and is considered somewhat at length.

*Case 1.*—Woman, age 24. Married and has one child. The patient had the usual diseases of childhood, most of them being very severe, but with no serious complications. Menstruation began at 13; during the first year the periods were irregular and accompanied by severe pain. Since then they have given her no trouble. No hereditary disease of any kind is known to exist in the family.

The patient gives a history of turbid urine since girlhood and years ago was told by physicians that she had "bladder trouble." About six years ago she noted that the urine was bloody after excessive physical fatigue and nervous strain. This disappeared in a few days, but a year later she had a recurrence following fatigue. Four years ago, at intervals of several months, she had four attacks of severe cramplike pains in the abdomen about the median line which required opiates for relief. No blood appeared in the urine at the time of these attacks, and no chill, fever, or vomiting accompanied them.

Three years ago the patient was married. Soon afterward she had an attack similar to asthma lasting for several weeks. A few weeks later there appeared in the urine, which had been turbid at intervals, white ropy streaks and on cystoscopic examination a probable diagnosis of renal tuberculosis was given and she was advised to go to California for the winter, which she did. While there she became pregnant and noted that at intervals the urine was bloody. The hematuria continued during pregnancy. A forceps delivery was necessary; the child was normal and no complications occurred. Following pregnancy the hematuria recurred at intervals as before and has continued up to the present time becoming, on the whole, more frequent and more

severe. During February, 1907, she took a severe cold and was in bed for several weeks during which time she lost much weight and strength. The hematuria at times was severe. A year ago, and also more recently, X-ray examination failed to reveal any evidence of stone in the urinary tract. Examination of the urine for tubercle bacilli by staining and also by inoculation of the sediment in guinea-pigs has constantly given negative results.

In June, 1907, she entered the Presbyterian Hospital in the service of Dr. Billings. The lower pole of the right kidney was easily palpable; otherwise physical examination revealed nothing abnormal. The blood gave 3,500,000 reds, 8,600 whites, and 57 per cent hemoglobin. Temperature normal. The urine was constantly turbid; some days it was bloody, at other times straw-colored. Red corpuscles together with a considerable number of leucocytes and bacteria were always present. Casts were never found. Albumin was present in small amount; no sugar.

A few days after entrance a cystoscopic examination was made by Dr. J. C. Webster and both ureters catheterized. The bladder was normal. A small clot of blood appeared at both ureteral openings. The right ureter appeared to be thickened and the catheter entered with considerable resistance but did not meet a definite obstruction. No urine was obtained from this ureter. Urine flowed freely from the left ureter. It was turbid, acid in reaction, contained a few leucocytes, red cells, epithelial cells, and a small amount of mucin; also bacteria identical with those obtained previously from the bladder and which will be described later.

A few days later a right nephrotomy was made. The capsule was normal. The pelvis and ureter were thickened and the catheter met an obstruction in the ureter a short distance below the pelvis. There was no evidence of tumor, calculi, or tuberculosis. Fluid from the pelvis of the kidney showed bacteria identical with those obtained from the bladder and the left ureter. A small piece of kidney tissue, removed at the operation, microscopically showed some degeneration of the cells and between many tubules the blood vessels were dilated. No blood was seen outside the vessels. The kidney was decapsulated, fixed, and drained. The tube remained in the kidney for several weeks and argyrol injected into the tube passed freely through the ureter into the bladder. The opening later closed and the tube was removed. A few days later blood appeared in the urine in quantity sufficient to color it distinctly red. For three weeks the urine was free from blood, then suddenly reappeared and has continued almost constantly. The patient's general condition remained about the same; the hemoglobin has remained low (about 60 per cent).

From the urine, from both ureters, and from the pelvis of the right kidney a colon-like bacillus was obtained in pure growth on the plates. Smears of the urine showed also a few Gram-positive bacilli often occurring in long chains. The colon-like bacillus was found constantly in the urine for several months. The ureters were catheterized just before the operation and again seven months later and this same organism obtained each time. No doubt the same bacillus has been the constant inhabitant of the urinary tract during many years.



The bacillus has the following properties:

Morphologically it resembles *B. coli*. It is a Gram-negative, non-motile bacillus, rather slender, and not infrequently forms short curved threads. On all media it grows less luxuriantly than *B. coli* and produces gas slowly in glucose with the ratio  $H:CO_2 = 2:1$ . It also produces gas in maltose, levulose, saccharose, lactose, and inulin. It permanently acidifies milk in 36 to 48 hours, but coagulation does not occur upon standing, even for many weeks. It produces indol and nitrites in Dunham's peptone medium; it grows freely in urine, causing uniform turbidity and later considerable sediment. It does not produce hemolysis on blood-agar plates. For animals it has about the same pathogenicity as *B. coli*. One agar slant kills a guinea-pig as a rule in 24 hours.

This bacillus, therefore, differing as it does from *B. coli*, in not coagulating milk and growing less luxuriantly on various media, may be considered a modified colon organism.

With a view to inoculation treatment, the opsonic index of the patient for this bacillus was obtained from time to time. Two determinations gave indices of 0.7 and 0.4 respectively (Chart 1). After the injection of 50 million bacilli killed by heat the index promptly rose. The injections were continued at intervals of about one week and the index, as shown in the chart, continued to remain high. Heated serum (Chart 1) and the dilution method also gave high indices, as a rule above 2, the curves on the whole corresponding very well. The difference between the phagocytosis in the normal and the patient's serum was so striking that it could readily be detected by merely inspecting the slides. The index for other bacteria was normal or slightly below (Table 1). The specificity of the opsonin

TABLE 1.  
OPSONIC INDEX OF PATIENT'S SERUM FOR OTHER BACTERIA COMPARED WITH THAT FOR  
AUTOGENOUS BACILLUS.

Organism	Index	Index for Autogenous Bacillus
Typhoid.....	1.10	2.60
<i>Streptococcus pyogenes</i> .....	0.96	1.30
<i>B. coli</i> .....	0.61	1.30
<i>Staphylococcus aureus</i> .....	0.70	1.85
Pneumococcus.....	0.93	1.85
Tubercle Bacillus.....	0.75	2.60
<i>B. coli</i> (from a case of pyelitis).....	1.30	2.20

is evident. Even for typical colon bacilli which differ but slightly in cultural characteristics the index is much lower.

In determining the opsonic index it was noted that when unheated serum was used there was distinct evidence of bacteriolysis in the



tube containing normal serum, whereas in the tube containing the patient's serum little or no such change occurred. Under the microscope the process of solution of the bacilli in normal sera could be readily observed, while the patient's serum was quite inert. Also on comparing the lytic property of normal and patient's serum by the plate method, it was found that the former rapidly destroyed the organism while the latter had little or no lytic power. Heating normal serum to 56° C. for 20 minutes destroyed the lytic effect and heating the patient's serum produced no change. Table 2 is arranged to

TABLE 2.  
LYSIS OF COLON-LIKE BACILLUS.

	NO. OF COLONIES AT INTERVALS			
	At Once	2 Hrs.	5 Hrs.	18 Hrs.
Normal Serum A	100	0	0	0
" " A	5,000	0	0	0
" " B } Unheated.....	4,000	2	0	0
" " C	4,000	0	0	0
" " D	3,500	80	0	0
Normal Serum A	5,000	4,500	5,000	8,000
" " B } Heated 56° 20 min.....	2,000	2,000	2,500	2,000
" " C	2,000	1,800	1,800	30
NaCl Solution.....	4,000	2,000	1,000	1,000
Feb. 10, Patient's Unheated Serum.....	5,000	3,500	500	...
" 24, " " " ".....	2,000	1,500	2,000	500
Mar. 3, " " " ".....	4,000	800	1,000	100
" 17, " " " ".....	2,000	2,000	2,500	400
Feb. 17, " Heated Serum.....	5,000	6,000	6,000	6,000
" 24, " " " ".....	2,000	2,000	1,000	2,000
Mar. 17, " " " ".....	2,000	2,500	2,000	600

show the effect of heated and unheated sera upon the growth of this organism. Four different normal sera were tested and all acted alike. The lytic power of the patient's serum toward this organism was tested at intervals for a period of six weeks with the same results. A small amount of normal serum reactivated for lysis the normal heated serum but not the patient's heated serum. It appears, therefore, that in the patient's serum there is little or no specific lytic amboceptor for this particular organism, while in normal serum this body is present.

The lytic effect of the patient's serum was tested upon typical colon and typhoid bacilli and compared with the normal sera in this respect (Table 3). The patient's serum is as lytic for these organisms as normal sera.

TABLE 3.  
LYSIS OF COLON AND TYPHOID BACILLI.

	NO. OF COLONIES AT INTERVALS			
	At Once	2 Hrs.	5 Hrs.	18 Hrs.
<i>B. coli</i> (typical) + Patient's Serum.....	6,000	1,500	75	0
<i>B. coli</i> " + Normal Serum A.....	8,000	3,000	200	46
<i>B. coli</i> " + " " B.....	8,000	4,000	600	10,000*
<i>B. typhosus</i> + Patient's Serum.....	3,000	3	0	0
" + Normal Serum A.....	4,000	5	0	0
" + " " B.....	2,000	15	0	0
" + " " C.....	6,000	900	200	0
" + NaCl Solution.....	6,000	3,000	2,000	1,000

\* This serum was obtained from a person who a few months previously had an operation for acute appendicitis. The hemolytic property of his serum was also less than normal.

The lytic power of the serum upon goat, rabbit, and guinea-pig corpuscles was normal.<sup>1</sup>

This serum differs, then, from normal serum in that it has practically no lytic effect upon the autogenous bacillus; it possesses, however, normal lytic power for other even closely related bacteria and also for red corpuscles.

Owing to the rapid lytic action of normal unheated serum upon the bacillus accurate determination of opsonin consequently could not be made. With heated sera this difficulty was removed, because, as shown, heated normal and patient's sera have practically no lytic power. Heating to 56° C. for 20 minutes markedly decreased, but did not entirely prevent phagocytosis in either normal or patient's serum.

In order to show more clearly the relation of phagocytosis to bacteriolysis in heated and unheated serum, the process was followed by making smears of mixtures in the incubator at frequent intervals and determining the amount of phagocytosis. Chart 2 shows a series of curves obtained in this manner. Counts were made at 3, 6, 10, 20, 60, and 120 minute intervals of the number of bacilli in 50 leucocytes.

Curve A is lytic normal serum. Phagocytosis proceeds rapidly in the first few minutes, but owing both to intra- and extra-cellular lysis the curve falls. The bacilli in the stained preparations were disintegrating both inside and outside the leucocytes and at the end of two hours had largely disappeared. Curves B, C, and D represent

<sup>1</sup> I have to thank Dr. Preston Kyes for these experiments.

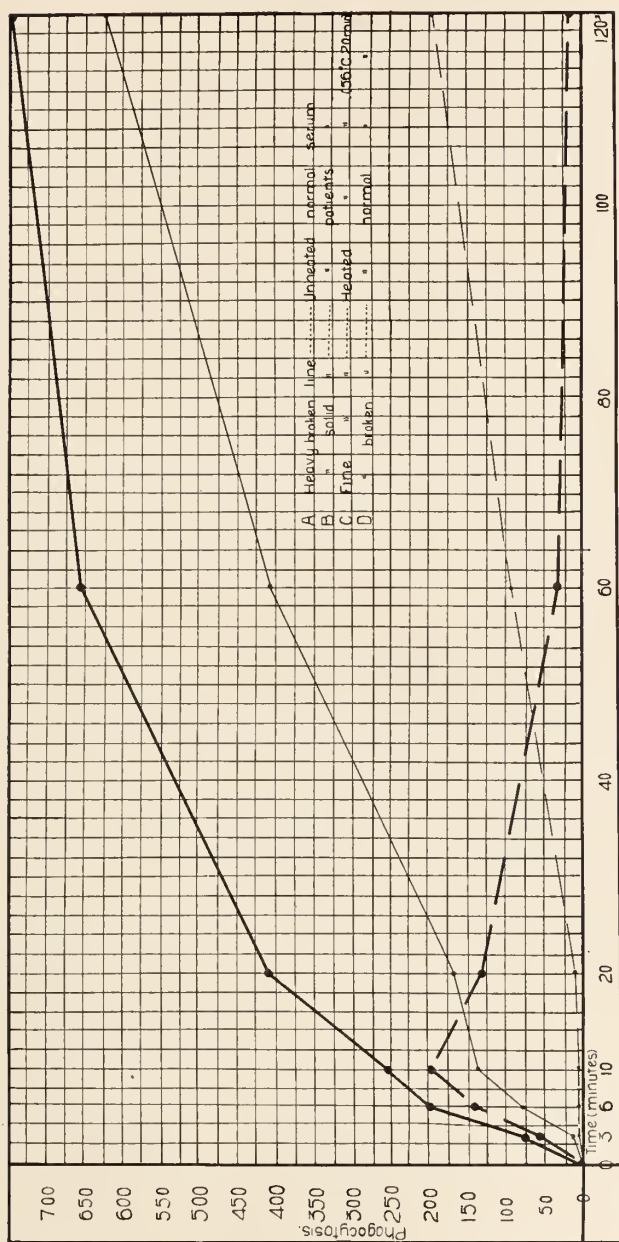


CHART 2.—The curves represent the average number of bacteria taken up by 50 leucocytes at varying intervals of time, using unheated and heated normal serum and serum of patient in Case 1.

the process of phagocytosis independent of bacteriolysis. The space between *B* and *C* represents the loss of opsonic effect by heat in the patient's (immune) serum. The space between the normal line and *C* represents the thermostable opsonic element. In heated normal serum the opsonic content was low as shown by curve *D*. At the end of 120 minutes, however, the phagocytosis is considerable. It is interesting to observe that here the bacilli within the leucocytes as well as those outside remained undigested, while in the unheated normal serum the digestion goes on rapidly, both within and without the cells. It would appear from this that intracellular digestion of the bacilli depends upon the action of the serum either upon the bacteria before phagocytosis occurs or upon the leucocytes. In curves *B* and *C* the leucocytes at the end of 120 minutes were well filled with bacilli which show only slight evidence of digestion. Undoubtedly intracellular digestion was going on, as will be shown later, but the process was so slow that phagocytosis kept the leucocytes well filled with bacilli but little injured so far as the staining reaction indicated.

This series of curves shows clearly the importance of the time interval in determining the opsonic index as well as the necessity of knowing whether or not a serum is lytic. For instance, in using unheated sera in this case the longer the interval of time the greater will be the opsonic index; with heated serum, since the curves are more nearly parallel, the index at varying intervals will not vary greatly. With lytic sera, especially unheated, indices obtained within the first few minutes will on the whole be much more trustworthy.

Reactivation experiments with the patient's heated serum and with normal heated serum gave definite results pointing to the co-operation of a thermostable and a thermolabile substance in opsonification. A small amount of normal serum added to the heated sera increased phagocytosis both in the patient's serum and in normal serum.

Table 4 shows the combined action of the two bodies in opsonification and also indicates that the thermostable body, not being removed by washing, is securely bound to the bacteria. The thermolabile body on the other hand is easily removed by washing. These facts hold true for both normal and immune serum.

The agglutinating power of the serum was tested upon the homol-



ogous bacillus at various intervals. These experiments were made at the same time as the dilution experiments for phagocytosis, every day or every second day for a period of two weeks. Both macroscopic and microscopic methods were used, but at no time was any specific agglutination observed. The bacillus was slightly agglutinated in dilutions 1-10 and 1-20 in both normal and patient's serum. In one or two instances the homologous serum seemed to be even less agglutinative than the normal.

TABLE 4.  
REACTIVATION OF HEATED IMMUNE SERUM.

1.	Bacilli	0.3 c.c. + Patient's heated serum	0.1 c.c. + NaCl Sol.	0.1 c.c.
2.	"	0.3 c.c. + " " "	0.1 c.c. + Normal Serum	0.02 c.c. + NaCl Sol. 0.08 c.c.
3.	"	0.3 c.c. + " " "	0.1 c.c. + NaCl Sol.	0.02 c.c.
4.	"	0.3 c.c. + NaCl	0.1 c.c. + Nor. Serum	0.02 c.c. + NaCl Sol. 0.08 c.c.

Incubated 45 minutes; centrifuged 30 minutes. Bacilli then suspended in 0.3 c.c. NaCl and mixed with leucocytes and serum as follows:

				Phagocytosis in 50 Leuco- cytes (20 minutes).
1.	Bacilli	0.3 c.c. + Leucocytes	0.1 c.c. + NaCl Sol.	0.1 c.c. .... 205
2.	"	0.3 c.c. + " "	0.1 c.c. + " "	0.1 c. .... 191
3.	"	0.3 c.c. + " "	0.1 c.c. + Nor. Ser.	0.02 + NaCl Sol. 0.08 c.c. .... 563
4.	"	0.3 c.c. + " "	0.1 c.c. + NaCl Sol.	0.1 c.c. .... 0

The importance of phagocytosis in destroying bacilli of this type was clearly brought out by the experiment, the results of which are shown in Table 5. The patient's unheated serum and washed

TABLE 5.  
INTRAPHAGOCYTIC DESTRUCTION OF COLON BACILLI.

	NO. OF LEUCOCYTES IN 1 CU. MM.	NO. OF COLONIES ON PLATES			
		At Once	2 Hrs.	5 Hrs.	18 Hrs.
1. Unheated Serum.....	.....	2,000	1,500	2,000	500
2. Washed Leucocytes.....	30,000	2,000	800	500	600
3. Washed Leucocytes + Serum.....	400	2,000	2,000	600	3,000
4. " " + ".....	4,400	2,000	200	600	5,000
5. " " + ".....	16,000	2,000	10	0	0

leucocytes by themselves have no prompt destructive effect on the bacilli. When mixed the result depends upon the number of leucocytes present. If this number is low (400) the bacilli are not destroyed; by concentrating the leucocytes (16,000) they are destroyed, thus showing that the bacilli after being taken up by the leucocytes are killed even though they microscopically show little evidence of intracellular digestion. In this connection may be mentioned some

experiments the results of which appear to indicate differences in the phagocytic activity of the patient's leucocytes and of normal leucocytes. Dudgeon and Shattock<sup>1</sup> found as a rule an increased activity of the immune phagocytes, but in certain conditions it was normal or subnormal.

Table 6 shows the result of crossing normal and patient's leucocytes with normal and patient's sera. Four experiments were made,

TABLE 6.  
COMPARATIVE PHAGOCYTIC ACTIVITY OF LEUCOCYTES.

					No. of Bacilli in 50 Leucocytes	
Normal Leucocytes	A	+ Patient's Serum	+ Bacilli		278	} 376
"	"	+ Normal	" A +	"	98	
Patient's	"	+ Patient's	" +	"	296	} 374
"	"	+ Normal	" A +	"	78	
Normal	"	B* + Patient's	" +	"	148	} 192
"	"	+ Normal	" B +	"	44	
Patient's	"	+ Patient's	" +	"	188	} 250
"	"	+ Normal	" B +	"	62	
Normal	"	C + Patient's	" +	"	134	} 174
"	"	+ Normal	" C +	"	40	
Patient's	"	+ Patient's	" +	"	254	} 446
"	"	+ Normal	" C +	"	192	

\* Experiment with leucocytes B repeated at another time gave substantially the same results.

using leucocytes from three different normal persons. The number of bacilli taken up by normal leucocytes A and patient's leucocytes were about the same. Normal leucocytes B and C contained less bacteria than the patient's leucocytes. This may be interpreted in one of two ways. The number of bacteria as determined by count is not necessarily the number taken up by the leucocytes because of the process of intracellular digestion which is an important factor in the case of this bacillus. Consequently the data given in Table 6 may signify either an increased phagocytic activity of the patient's leucocytes or a less rapid process of intracellular digestion in these leucocytes as compared with normal leucocytes or both.

*Case 2.*—This case was a chronic urinary infection of at least five years' standing, occurring in a young man. The urine was constantly turbid, contained leucocytes in small numbers but never any blood. A short, plump, non-motile, Gram-negative bacillus was obtained in pure growth; it produced gas readily in the various sugars, acidified but did not coagulate milk, and formed indol. Injections of the autogenous dead bacilli in doses of 400 million to one billion were given every 5 to 7 days. They

<sup>1</sup> *Lancet*, 1908, 86, p. 618.

gave rise to definite local and general reactions, manifested by localized tenderness, redness, and swelling at the point of injection, and by fever, leucocytosis, irritation of the bladder, malaise, and soreness in the muscles and joints generally.

The opsonic index (Chart 3) was high at first and rose still higher following the inoculation treatment. As a rule a drop occurred in

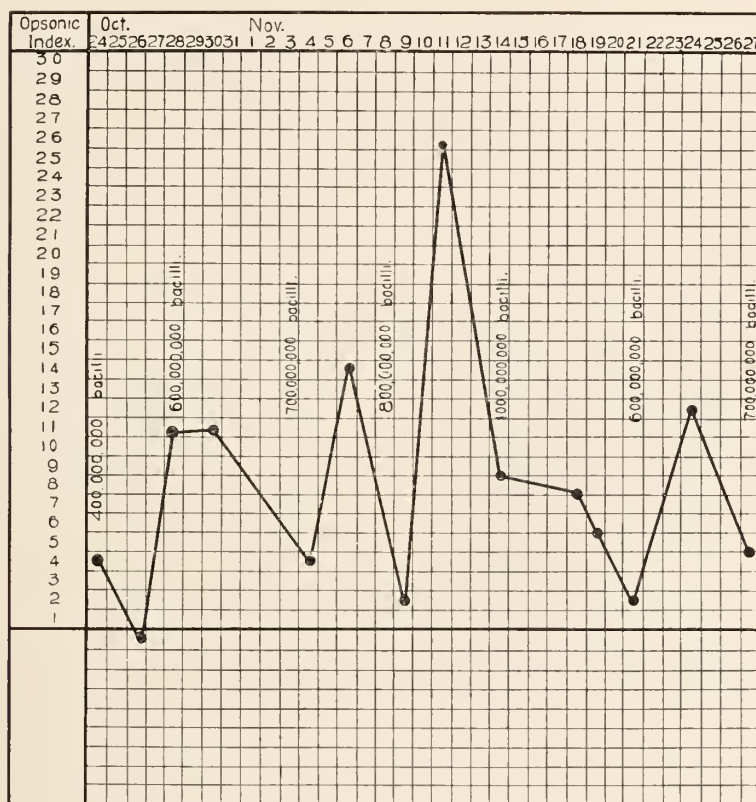


CHART 3.—Opsonic index of Case 2 obtained with unheated serum and homologous bacilli.

the opsonic curve after the injection, followed by a marked rise. A striking feature was the comparative insusceptibility of this organism to phagocytosis, necessitating the use of dense suspensions of bacteria to obtain an appreciable phagocytosis. The organism was not highly virulent for animals. The opsonic index for another strain of *B. coli* was normal.

Lysis of this bacillus was clearly evident under the microscope. By the plate method it was shown that while both were distinctly lytic, normal serum was more so than the homologous serum. This continued so in spite of repeated injections with the dead bacilli. In marked contrast to Case 1, serum heated to 56° C. for 20 minutes seemed to lose its opsonin entirely for this bacillus. Tests made over a period of several weeks gave uniform results. By adding small amounts of normal serum to the heated sera it was possible to reactivate both the patient's serum and normal serum for opsonification. For bacteriolysis slight reactivation was obtained in both normal and patient's sera.

*Case 3.*—In this case, which was one of long-standing infection of the urinary tract with a typical colon bacillus, probably associated with stone, the opsonic indices, taken five times on alternate days, were 0.4, 0.2, 0.2, 0.6, and 0.7. Using heated sera they were approximately the same. The lytic power of the serum taken twice during this period was practically normal. Here the unusually low opsonic content was associated with the normal amount of lytic substance. No inoculations were made.

*Case 4.*—For the last two months of pregnancy the patient had turbid urine, frequent urinations, and chills and fever. A pure growth of a colon-like bacillus was obtained from the urine associated with a large number of leucocytes. The opsonic index was about normal. An injection of 500 million dead bacteria was given. At this time birth occurred. The indices on the following alternate days were 1.8, 3.0, 3.5, 4.3, 2.0, 8.5, 2.1, 3.8, and 3.3. The bacteriolytic action of the serum was normal.

*Case 5* was one of urinary infection of several months' standing with a non-hemolytic, Gram-negative, non-motile bacillus which acidified and coagulated milk and fermented the various sugars with the production of gas. The opsonic index taken at three different times was 1.1, 1.3, and 1.2. The bacillus was equally susceptible to the lytic action of both normal and homologous sera, even in very high dilutions. No specific agglutination was observed.

*Case 6* was one of long-standing urinary infection occurring in a man who gave a history of syphilis 22 years back and gonorrhea 15 years back, complicated with stricture. Eighteen months before coming under observation the patient developed a more or less general acute painful arthritis involving especially the right foot, and later both elbows, hands, and the left foot. He also had symptoms pointing to locomotor ataxia and from the prostate were obtained forms suggestive of gonococci. The temperature was as a rule normal. The urine was constantly turbid and contained numerous pus cells and bacilli. Urination was frequent and difficult. The organism present in the urine was a Gram-negative, non-motile bacillus often slightly curved and staining irregularly. Phagocytosis was not appreciable in the urine but occurred freely in the presence of serum. It did not acidify or coagulate milk, produced a trace of indol, fermented mannite and dextrin, but not glucose or galactose. In aerobic cultures growth as a rule was scant, both in plain and special media, and at times no growth whatever occurred. In anaerobic cultures growth always took place and was much

more profuse. On blood plates the colonies were from 0.5 mm. to 1 mm. in diameter, surrounded by a wide clear zone of hemolysis. There was no odor.

Both normal and homologous sera were equally highly lytic for this bacillus while salt solution was inert. Slight agglutination occurred in two hours at 1-40 in the patient's serum but none in the control. The opsonic index taken from time to time varied from normal to 2 or slightly above.

For a period of many months the patient received injections of the dead bacilli about once a week in doses varying from 200 million to one billion. He received two injections of dead gonococci early in the treatment. A slight reaction occurred. The reactions following the injection of bacilli were at times intense. Marked improvement took place the details of which will be discussed more fully later. Here attention is called to the anaerobic character of the bacillus, its marked susceptibility to lysis by both normal and homologous sera, and the slight agglutinating property of the patient's serum, its opsonic power being higher than normal.

*Case 7* gave a history of an indefinite "grippe" infection several months previously. Improvement followed, but later there developed aching pains and stiffness in various joints, particularly the knee. No marked swelling of the joint occurred at any time and no urinary symptoms were noticed. The urine (catheterized specimen) upon examination showed the presence of a small number of pus cells, some epithelium, but no red cells or casts. A very small, irregularly staining, Gram-negative, non-motile bacillus was found in smears made from the sediment and on blood-agar plates small colonies appeared after from 48 to 72 hours. These were so small that they might easily escape detection on the plates were it not for the clear wide zone of hemolysis about them. At times in aerobic cultures no growth whatever occurred in the plates. Under anaerobic conditions, however, the growth was more rapid and profuse, and as a rule produced a wider zone of hemolysis. All attempts to grow this organism on media other than blood media were futile. The organism was readily killed by heat, and about once a week from 100 to 500 million bacilli were injected subcutaneously. A definite reaction followed, manifested by local swelling, tenderness, redness, slight fever, and leucocytosis with increased pains in the joints and muscles generally. In this case no vesical irritation followed these injections as occurred in some of the other cases. Marked improvement took place, the bacilli gradually decreased in numbers in the urine, and the pains and stiffness in the joints improved. The bacilli growing in clumps both in the urine and in media, attempts to determine the opsonic index were not satisfactory. The percentage index gave 0.66 and 1.8 at two different times. In this case considerable spontaneous phagocytosis occurred much the same as in the case of influenza bacilli.

*Case 8* is one of long-standing tuberculosis of the genito-urinary system with secondary colon infection in a man, 31 years of age. Eight years previously the infection began as an acute painful swelling of the right testicle. Later the glands in both groins became involved. These were opened and drained and the testicle removed. For two years the patient did not do well, and after two further operations he went to New Mexico where he improved greatly. But the following year there was a recurrence of the disease in the groin, and a year later he began to have pain in the small of the back on both sides and also pain on urination. At first the urine was clear but soon it became turbid and he was obliged to urinate frequently. Up to this time his general health remained fairly good. Now, however, he rapidly lost in strength and weight and remained in bed a large part of the time. The pain in the back and in the region



of the bladder was intense and he was not able to lie on his side. In the spring of 1907 right nephrotomy was made but no improvement took place. In the fall of 1907 he came to the Presbyterian Hospital in the service of Dr. Billings. The urine contained constantly a large amount of pus, often considerable quantities of blood, and numerous tubercle bacilli. Urination occurred every 30 minutes and was accompanied by severe pain. Walking, sitting, and lying on either side were painful and he spent most of the time on his back. The ophthalmo-tuberculin test gave a positive reaction. In addition to the tubercle bacilli there was an abundant growth of a typical colon bacillus in the urine. Simultaneous injections were given of Koch's new tuberculin (1/1,000 mgm.) and of killed autogenous colon bacilli. The tuberculo-opsonic curve followed a fairly typical curve, showing a slow but gradual rise in response to the injections. Injections of the dead colon bacillus in doses of 500 million were given once a week and caused sharp reactions both local and general; after three injections the bacilli had entirely disappeared from the urine. The colon opsonic index which was low at first (0.8) rose to between 2 and 3 and remained high. The patient gradually improved. The colon injections were discontinued and the tuberculin given weekly continuously for a year. During this time the patient gained over 20 pounds in weight and became much stronger in every way. The pains grew less, urination occurred about once in two hours, and he was able to go about and do light work. One year later his tuberculo-opsonic index was 2.1 and the colon bacilli were not found in the urine. Tubercle bacilli were still found and pus cells were numerous, though the centrifuged sediment was not one-tenth as much as a year previously. The opsonic and temperature curves are given in Chart 4.

#### SUMMARY AND GENERAL CONSIDERATIONS.

In a case (Case 1) of long-standing infection with a colon-like organism the specific opsonic index, low at first, upon inoculation rose and remained high. The patient's serum had no bacteriolytic power for the homologous bacillus and no change in this respect followed the inoculations. Normal sera for the same organism were highly lytic. For other closely related bacilli (*B. coli* and *B. typhosus*) and for red corpuscles of various animals, the patient's serum was normally lytic. It was possible to reactivate the patient's heated serum for opsonification but not for bacteriolysis, while normal heated serum could be reactivated for both opsonification and bacteriolysis. Specific agglutinins were not present in the patient's serum.

In a second case (Case 2) a high opsonic content was associated with a subnormal lytic power. Normal serum and the patient's serum could be reactivated for both bacteriolysis and opsonification. Specific agglutinins did not appear.

In a third case (Case 3) an unusually low opsonic index was associated with normal lytic activity.



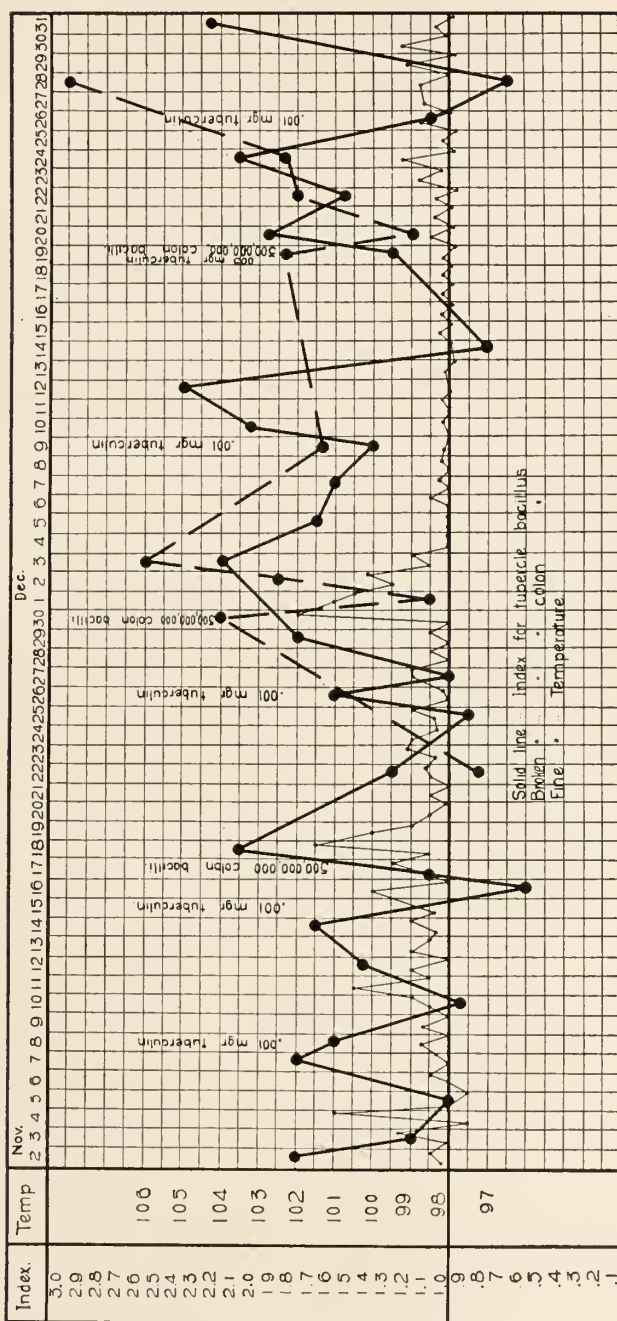


CHART 4.—The temperature curve and opsonic index for tubercle bacillus and colon bacillus in Case 8.

These facts appear to be important in relation to the question of the identity of the lytic amboceptor and the stable opsonic body. In Cases 2 and 3 one may explain the results by either one of two assumptions. First, one may assume that opsonins and lysins are different substances and the one may vary independently of the other; or, second, the opsonin and lysin may be one and the same and on account of certain changes the microbe has become immune to bacteriolysis or opsonification as the case may be. Either of the above assumptions may explain, perhaps equally well, the facts as observed in these two cases, but they do not explain equally well the facts in Case 1. Here the serum increased markedly in its specific opsonic property as a result of inoculation, whereas it remained as inert as salt solution in its specific bacteriolytic power, while the bacillus was highly susceptible to bacteriolysis by normal serum. On the basis of the first assumption, namely, that lysins and opsonins are different substances and may vary independently, the facts may be explained thus: the opsonins increased and remained high; the lysins normally present when the infection began were absorbed and for some reason the body did not react to produce them in appreciable quantities. On the basis of the second assumption—that opsonification and bacteriolysis depend on the same substance and the microbe undergoes specific changes—it is impossible to understand how the bacillus could acquire the property of resisting bacteriolysis and at the same time not only retain but vary in its susceptibility to phagocytosis as the facts indicate. We might say that in Case 1, for example, the bacillus had immunized itself against the lytic action of the patient's serum. But from what is known of the principles of immunity it should also be immune to the lytic action of serum from all individuals of that species. This is not true.

The facts are better explained by assuming that the microbe gaining access to the individual causes specific reactions in the serum to meet which the microbe specifically adapts itself. The microbe thus immunizes itself against an abnormal individual and may be still susceptible to the serum of a normal person. This reciprocal adaptation continuing for a long period may give rise to profound changes on the part of both microbe and host. Attention is called to the fact that the cases here reported are very chronic, Case 1, for

instance, dating back at least five years and possibly ten or more years. It would not be surprising, therefore, to meet with changes in the serum in such cases, very different in character from the changes ordinarily met with in the more acute infections.

#### OPSONINS IN THE URINE.

Normal urine when added to washed leucocytes and colon bacilli does not cause phagocytosis. If serum is added to this mixture the leucocytes take up the bacteria as freely as if salt solution replaced the urine. Urine, therefore, does not inhibit phagocytosis. In Case 1 there were constantly present in the urine, even when blood was absent, numbers of polynuclear leucocytes and albumen, but phagocytosis occurred only to a slight extent even when blood was present in large amounts and the bacilli formed a dense suspension. In the test-tube bloody urine added to washed corpuscles and bacteria and allowed to incubate for one hour caused an appreciable amount of phagocytosis. An experiment made with a specimen of such bloody urine showed slightly less phagocytosis than in a 1:100 dilution of the patient's serum taken at the same time. In Case 2 the urine, which was free from blood and albumen, was tested for opsonin at a time when the opsonic index of the blood was very high (11.1). A few leucocytes and many bacilli were present in the urine but there was no appreciable phagocytosis, either at the time it came from the bladder or upon standing in the incubator. Serum added to such urine caused the leucocytes to freely ingest the bacteria. These leucocytes, however, were not as active as fresh leucocytes obtained from the blood and many were undergoing disintegration. It appears, therefore, that the urine, from certain cases whose blood possesses a high opsonic content for the infecting organism, may contain practically no opsonin for the same organism. On the other hand at times, as was noted in certain of the cases, an appreciable phagocytosis of bacilli occurred in the urine. In the cases in which this was observed, however, the bacilli were far more phagocytatable than the bacilli from the cases mentioned above.

In Case 8 the urine contained constantly large numbers of tubercle bacilli, but it was exceptional to find one within a leucocyte. At times there seemed to be slightly more phagocytosis than at others.

After marked improvement had occurred and the tuberculo-opsonic index was high (2.1) the relation of bacilli and leucocytes did not change. These findings are in accord with those of Dudgeon<sup>1</sup> and also with those of Opie<sup>2</sup> who found frequently little or no opsonin in many inflammatory exudates.

#### INOCULATION OF DEAD COLON BACTERIA AND THE REACTION PRODUCED THEREBY.

In view of the fact that the bacilli isolated from cases of urinary infection usually differ more or less from one another, homologous bacilli were used in the cases here reported. The bacilli in pure growth were killed by heating to 60° C. for 30 minutes. This was sufficient in every instance to kill the bacilli as shown by the control culture. Fresh suspensions should be used. For the first injection not over 200 million bacilli should be given; this may then be increased until a suitable reaction is obtained. It is necessary to do this for the reason that the strains isolated from the various cases differ in the strength of reaction which they produce. Three hundred million bacilli of one strain may produce as strong reaction as 600 million organisms of another strain. As has been stated the bacilli of various strains differ considerably in their size and this may partly explain variations in effect when the number of organisms is used as a standard. Other factors also undoubtedly come in. The dose, therefore, for organisms of this type may vary from 200 million to one billion.

The local reaction consists of tenderness, redness, and swelling over an area several centimeters square. This begins in one or two hours following the injection, reaches the height in about 12 to 18 hours, and disappears in 48 to 72 hours. A general reaction occurs, manifested by leucocytosis, fever, sometimes headache, general malaise, aching in muscles, bones, and joints, and often, but not in all cases, irritation of the bladder indicated by a frequent desire to urinate. This latter symptom was especially marked in Case 2. Because of the fact that the strains of bacilli vary so markedly in their cultural properties, and especially in their serum reactions, it does not appear reasonable to use stock solutions of dead colon bacilli for purposes of treatment or for diagnosis.

<sup>1</sup> *Loc. cit.*

<sup>2</sup> *Jour. Exp. Med.*, 1907, 9, p. 515.

It would be folly to attempt to draw definite conclusions concerning a therapeutic question from the series of cases here given. Suggestions only may be made and the facts taken for what they are worth. In one case only (Case 8) did the bacilli entirely disappear from the urine while the case was under observation. In most instances the condition of the urine improved as indicated by the number of leucocytes and bacilli, but did not entirely clear up, at least while the patient was under observation. The result in Case 6 was striking. The patient, following injections of about 500 million dead bacilli, went about his work. An hour later he had a most violent chill, followed by high fever ( $105.4^{\circ}\text{F.}$ ). After 24 hours the temperature became normal and the patient was able to go about as usual. This appeared to be the turning-point in his case. Severe and almost constant pain which he had had in his ankles for over a year appeared to have left him during the time he had the severe reaction, to return, if at all, only in very mild form. He quite rapidly improved, especially as to the arthritis, in a short time gained 30 pounds, and felt better than he had for years. The urine improved but did not entirely clear up. Injections were continued for nearly a year and the case is still under observation. Case 4 quickly improved after childbirth and the inoculations probably had little to do with the result.

In the cases which show a diminished bactericidal power of the blood, such as Cases 1 and 2, and also those with low opsonic indices, such as Case 3, it appears reasonable to attempt to combat the infection by using a method which will tend to raise the content of these immune substances in the body. In Case 1, for instance, where there was such a marked diminution, or a total absence of lytic substance, long-continued injection, though raising the opsonic index markedly, did not appear to have any appreciable affect in increasing the lytic power of the serum. This agrees with the results of Dudgeon who found that the opsonic indices were, as a rule, markedly affected by dead colon bacilli but anticolon serum had no more specific bactericidal effect than normal serum.

#### CONCLUSIONS.

In cases of urinary infections the bacteria of the colon group vary markedly in certain details. Consequently, for inoculation treat-

ment in these cases, the homologous germ should be used. The opsonic index may be raised by such inoculations. In certain cases the patient's serum may show a marked diminution or total absence of lytic bodies for the homologous bacilli, as compared with normal serum, while the specific opsonins may be present in abundance. In certain cases opsonins seem to play little or no part in the destruction of colon bacilli in the urine.

Opsonification, bacteriolysis, and agglutination in certain cases do not run parallel. The facts are most easily explained by assuming the existence of specific opsonins, lysins, and agglutinins.

NOTE.—Since this paper was written further results in some of the cases referred to and also in other cases treated later appear distinctly to favor the inoculation treatment. In Case 2 in which the inoculations were given weekly for nearly four months the pus and bacilli which were present in the urine constantly for over five years have entirely disappeared and the patient is now perfectly well. Case 1, clinically, has improved very much, but it is not known whether the bacilli have entirely disappeared. Case 6 improved markedly for a time but urethral strictures and symptoms of beginning locomotor ataxia have interfered with the results. A case of subacute pyelitis caused by a hemolytic colon-like bacillus apparently responded promptly to the inoculations and after five injections the urine was entirely free from pus and bacilli. Several other chronic cases now under observation all report improvement. Further detail of the treatment of these cases will be given later.



# IMMUNOLOGICAL AND EXPERIMENTAL STUDIES ON PNEUMOCOCCUS AND STAPHYLOCOCCUS ENDO- CARDITIS ("chronic septic endocarditis").\* †

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## INTRODUCTION.

THE factors which determine the localization of bacteria upon the endocardium in endocarditis and their maintenance there are still obscure. In the present article are recorded the results of observations upon the immunological reactions of bacteria isolated from cases of endocarditis, and upon experimental endocarditis. These results indicate that the production of endocarditis by staphylococci and pneumococci, as well as its character and course, depends to a greater degree than heretofore known upon certain acquired and peculiar properties of the bacteria in question.

STAPHYLOCOCCUS WITH SPECIAL CHARACTERISTICS FROM A CASE OF ENDOCARDITIS.

**Case 334.**—Salesman, 36. No history of previous infection; no rheumatism. Five months previous to beginning of present illness there was ulceration of first right lower molar tooth which was treated by a dentist. The tooth was crowned but the crown had to be removed a month later to let out pus and was soon replaced without further trouble until a week before death.

January 23, 1908, the patient first consulted Dr. Slaymaker for an acute attack of rhinitis and pharyngitis with severe muscular pains, backache, and prostration. A probable diagnosis of influenza was made, but the patient grew worse and entered the hospital four days later. At that time the spleen was palpable but there was no

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agglutination of typhoid bacilli by the serum. No heart murmur was detected. The leucocyte count was 7,950, four days later 12,380; hb. 76 per cent. No malarial parasites. The urine was normal.

February 9, 1908: Severe pain over kidneys and right side posteriorly requiring morphine for relief. Urine previously normal now contains albumin and a large amount of pus, many leucocytic, granular, and blood casts. There are numerous small petechial hemorrhages over the skin associated with a distinct systolic murmur transmitted to the left.

From this time the patient rapidly failed, the picture being that of a rapidly increasing infection. Three days before death he developed a left hemiplegia which improved the day before death. The petechial hemorrhages in the skin became so numerous that a large part of the body was covered with them. Conjunctival hemorrhages also were present. Two blood cultures<sup>1</sup> during life and cultures after death gave a peculiar staphylococcus which is described later. The pulse, temperature, opsonic index, and leucocyte counts illustrate well the rapidly increasing infection.

*Anatomic diagnosis.*—Alveolar abscess; acute mitral ulcerative endocarditis; embolism of the mesenteric artery with mycotic aneurysm; thrombosis of the splenic vein; multiple infarcts of the spleen and kidneys; cloudy swelling and fatty degeneration of the liver, myocardium, and kidneys; icterus; anemia and emaciation; multiple hemorrhages of the skin, conjunctivae, serous and mucous membranes, endocardium, myocardium, adrenals, and kidneys; hyperplasia of the mesenteric lymph glands and spleen; lobular pneumonia; emphysema of the lungs.

The body is 178 cm. long; poorly nourished. The skin is pale, icteric, still warm. The sclera show numerous punctate and larger hemorrhages. The right first molar is loose and from the alveolus exudes upon pressure a grayish-white pus. The lips are studded with numerous punctate hemorrhages. The genitalia are normal. No edema of the extremities. In the skin everywhere are innumerable pin-head to pea-sized hemorrhages, usually discrete, but at places forming large confluent areas as over the inner surface of the right arm where the area measures 10 cm. across. In the peritoneal cavity a small amount of blood-tinged fluid. The abdominal organs are in normal position, no adhesions. There are numerous petechial hemorrhages in the peritoneum. The mesenteric lymph nodes are enlarged.

The organs in the neck not examined. Mucous membrane of the trachea and the bronchi red and studded with numerous small hemorrhages. Both lungs are free and voluminous, the rounded margins highly emphysematous. The pleurae are smooth and shining. In the right lower lobe, near the interlobular fissure, is an area of consolidation the size of a hen's egg, red and bloody on the cut surface. The upper and middle lobes are edematous. The left lung crepitates throughout except in an area 1 cm. × 8 cm. at the lower border of the lower lobe which is dark gray and moist.

<sup>1</sup> *Blood cultures in endocarditis.*—In this series the blood cultures were made in the usual manner by puncturing one of the large veins at the bend of the elbow and inoculating media with blood. Practically all observers have obtained a higher percentage of positive results in pneumonia, typhoid fever, and septic processes generally by the use of fluid than by the use of solid media. In endocarditis, on the other hand, at least of the type under consideration, agar media have given positive results repeatedly when the cultures in broth and milk remained sterile. For this reason and also because the latter method is a means of learning the number of bacteria in the blood, the importance of inoculating both liquid and solid media in all cases where endocarditis is suspected must be emphasized. Repeated cultures in a number of cases showed that while the bacteremia is constant the number of bacteria is never great. The number of colonies varied between 4 and 2,000 per c.c. of blood.

There is no edema in this lung. The tracheo-bronchial lymph nodes are dark red, soft, and enlarged.

Both visceral and parietal layers of the pericardium are studded with numerous small hemorrhages. There is an irregular cauliflower-like, whitish-gray growth on the mitral valve which plugs the greater portion of the orifice. There are no necrotic areas on the surface of the vegetation. The wall of the left ventricle measures 1 cm. in thickness. The myocardium is soft and yellowish. The tricuspid and semilunar valves are normal. The endocardium is studded everywhere with minute hemorrhages which are very numerous throughout the myocardium as well. The intima of the thoracic and abdominal aorta normal.

The spleen weighs 370 gm. The external surface is smooth, light red except over the many infarcts. The parenchyma soft. The inferior branch of the splenic artery is thrombotic for 1 cm.

Esophagus normal. Stomach moderately dilated; mucous membrane pale red, several ecchymoses in the fundus. Intestinal mucosa shows a number of small as well as large ( $\frac{1}{2}$  cm. sq.) hemorrhages.

The liver weighs 1,610 gm., externally grayish brown, more or less granular. Lobular markings distinct. The gall bladder normal. Pancreas normal. The right adrenal weighs 11 gm., the left 7 gm., and both are covered with numerous ecchymotic spots. The kidneys weigh 530 gms. together; the capsules adherent in places. Numerous minute hemorrhages throughout both kidneys. There are also numerous sharply circumscribed, whitish-yellow areas, some of which project from the surface in wedge-like form. The cortex and medulla are sharply defined, the cortex from 6 to 10 mm. thick. Numerous hemorrhages in the pelvis and ureters; only a few in the bladder. Genitalia normal.

The mesenteric branches supplying 80 cm. of the lower portion of the ilium contain an aneurysm as large as a hazel nut filled with thrombus.

*Bacteriologic examination.*—There were examined the blood from the heart, the vegetations on the mitral valve, the bile, the cerebro-spinal, pericardial, and peritoneal fluids, and pus from the ulcerated tooth. The heart's blood and vegetations gave a Gram positive coccus, single, in small and large clumps. The bile gave a short Gram negative bacillus. The cerebro-spinal and peritoneal fluids were sterile. The pericardial fluid gave a pure culture of the staphylococcus isolated from the blood during life. The pus gave *Staph. albus*, *Strepto. pyogenes*, *B. coli*, and a Gram positive bacillus. The staphylococcus from the pus of the tooth acidified and coagulated milk and grew on the surface of agar and in broth and other media exactly as does *Staph. albus*. The characteristics of the strains isolated from the blood on two occasions during life were exactly the same as those of the one isolated after death.

*Description of the staphylococcus isolated from the blood.*—Upon all media this coccus is of the same size as the pyogenic cocci. It occurs singly, in pairs, and in large clumps. It stains rather slowly with Löffler's methylene blue. It is positive to Gram's stain.

The surface colonies on agar plates are grayish white. They are more elevated, more granular, and more opaque than the colonies of *Staph. albus*. The deep colonies are elongated and opaque. On blood agar a narrow zone of hemolysis surrounds the colonies. Agar streak (aerobic) is grayish white with irregular elevated margin, sticking tightly to the surface so that it is necessary to tear the surface to dislodge the colonies. (This property was lost on cultivation.) Anaerobic

robically the growth is apparently equally rapid but not so opaque and does not stick to the surface of the medium. In ordinary broth there is a scum on the surface and tenacious, flocculent sediment at the bottom; the intermediate fluid being perfectly clear. Clumps of bacteria adhere closely to the side of test-tube; vigorous shaking fails to dislodge them. After 48 hours the sediment in the bottom may fill half the tube, the media gradually becoming cloudy. After a week the special properties are greatly diminished, and soon they entirely disappear, so that six months later broth cultures produced a diffuse turbidity from the beginning. The anaerobic 48-hour broth culture shows a diffuse turbidity, no scum, only a very small sediment, and no clumps adherent to the sides of the tube. For one month this coccus did not liquefy gelatin when grown aerobically. It liquefied gelatin slowly from the beginning when grown anaerobically. Six months later it liquefied slowly along the line of inoculation. In litmus milk its growth is abundant when grown aerobically but no acid production could be demonstrated until after cultivation for six weeks. After that the milk turned to pink at the end of a week. At present, six months later, the

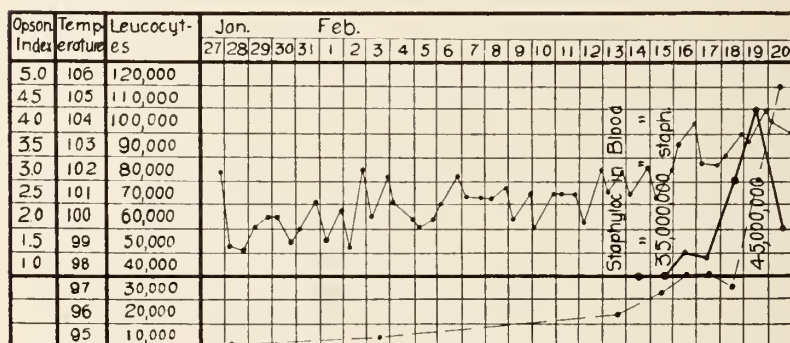


CHART 1.—Opsonic Index, Temperature, and Leucocyte Curves in Case 334. Solid heavy line = pneumococco-opsonic index; solid fine line = temperature; broken fine line = leucocytes.

acid production while still moderate is more marked than formerly. Anaerobic cultures gave moderate acid production from the beginning. The growth on potato and in glucose agar resembles closely that of *Staph. albus*.

*Microscopic examination.*—In sections of the lung there are patches of bronchopneumonia, some of considerable extent. In the liver are small regions of fatty changes. The kidney and spleen show infarction and in the myocardium there are scattered minute foci of inflammation.

The observations on Chart 1 and in Tables 1, 2, and 3 concern the foregoing case. The chart gives the pulse, temperature, opsonic, and leucocytic curves. The extremely high leucocyte count of 120,000 was composed of over 90 per cent polymorphonuclears. The opsonin curve should be followed in connection with the injection of killed staphylococci. The injection of 35 million ordinary staphylococci (*albus*) apparently caused a gradual rise in the index to 4.5 upon the





hand, the organisms obtained from the vegetations are susceptible to phagocytosis in the patient's defibrinated blood. Smears made directly from the vegetations show also evidence of phagocytosis.

TABLE 2.

OPSONIC EFFECT OF SERUM 334 AND NORMAL SERUM UPON STAPHYLOCOCCUS 334.

Mixtures (All contained equal parts of staphylococcus suspension, washed normal blood [washed three times in 40 times its volume], and serum or NaCl solution)		Phagocytosis
1.	Coccus 334 grown in Serum 334 48 hrs.	0
2.	" " " " heated* " "	7
3.	" " " " normal serum 48 hrs.	0
4.	" " " " heated " "	2.4
5.	" " " " Serum 334 and washed + NaCl Sol.	0
6.	" " " " " " " " + normal serum	10
7.	" " " " " " " " + Serum 334	0
8.	" " " " " " " " "heated & washed + NaCl Sol.	0
9.	" " " " " " " " " " + normal serum	6.8
10.	" " " " " " " " " " + Serum 334	3.5

\*Heated means 65° C. for one hour. The organisms designated as "washed" were washed twice in NaCl solution.

Table 2 summarizes the results obtained by a closer study of this interesting phenomenon. Lines 1 to 4 show that the acquired resistance of the coccus to phagocytosis is closely related to certain heat-sensitive properties of the serum as it resists phagocytosis completely when grown in the unheated serum, but becomes quite freely susceptible when grown in the heated serum, then suspended in washed normal blood and NaCl solution. Line 8 shows that the washed organisms grown in heated serum are insusceptible to phagocytosis when suspended in NaCl solution. The resistance acquired by the coccus when grown in unheated serum seems specific for the opsonins in this serum, because in normal serum it is taken up readily but not in the patient's serum (see lines 5, 6, 7). This is borne out by the results in lines 9 and 10 which show that when grown in heated serum the organism is susceptible to phagocytosis both in normal serum as well as in the patient's. Here we may have the explanation why a coccus of such a mild degree of virulence could live in a body and ultimately produce death, namely, an acquired, specific immunization against the antibodies of the host.

Table 3 shows that normal unheated serum has a bacteriolytic effect on staphylococcus 334 which is destroyed by heating to 60° C. for one hour. On the other hand the coccus grows as well in the unheated as in the heated serum of the patient. The staphylococcus aureus grows equally well in both sera. The patient's serum has a



marked agglutinating effect on strain 334 as have the sera from cases of pneumococcus endocarditis on homologous pneumococci.

TABLE 3.

EFFECT OF NORMAL SERUM AND SERUM 334 UPON STAPHYLOCOCCUS 334 AND *Staph. Aureus*

MIXTURES	AGGLUTINATION	COLONIES ON AGAR PLATES		
		At Once	12 Hrs.	48 Hrs.
Unheated normal serum + Coccus 334	No Agglutination	2500	1350	25
Unheated Serum 334 + " "	Marked "	3000	2800	5000
Heated * normal serum + " "	No "	2300	1800	3100
Heated * Serum 334 + " "	Moderate "	3000	2500	6300
Unheated normal serum + <i>Staph. aur.</i>	No "	350	∞	∞
Unheated Serum 334 + " "	Slight "	365	∞	∞

\* 60° C. for 1 hr.

∞ Innumerable.

The infection in this case undoubtedly came from the ulcerated tooth. Probably the gradual acquirement of the special properties described, namely, resistance to phagocytosis, adherence to surfaces, and growth in clumps, favored involvement of the endocardium. That the peculiar properties were acquired before the endocardium became involved and that the coccus immunized itself gradually against the antibodies in the patient as the infection progressed are indicated by the fact that as soon as these special properties were lost on artificial cultivation, it was impossible any longer to produce endocarditis experimentally; furthermore, repeated inoculations, instead of producing an immunity, produced hypersensitiveness and the coccus grown in one animal receiving repeated inoculations resisted phagocytosis and formed clumps. The fact that the organism grew aerobically in characteristic manner, but not when under anaerobic conditions, may be the reason why the left side of the heart was involved and not the right side. Intravenous inoculations of cocci grown anaerobically failed to produce lesions in three rabbits soon after the strain was isolated, an indication that the special characteristics were responsible for the promptness with which the coccus produced endocarditis so long as they were present.

## ANIMAL EXPERIMENTS WITH STAPHYLOCOCCUS 334.

Table 4 gives the most important results obtained in 12 rabbits inoculated from 5 to 15 days after the organism was isolated; six developed either a pericarditis or endocarditis or both. One of these six was inoculated directly into the heart, three intravenously; one received an intraperitoneal injection and six days later the second injection intravenously; and the other a primary intravenous and two weeks later an intraperitoneal injection. Three of the six that developed no lesions were inoculated

subcutaneously, one intravenously and two intraperitoneally. The two last received two intraperitoneal injections, recovering promptly after the first but succumbing to an acute fibrinous peritonitis after the second. Of the seven guinea-pigs none died after the first inoculation. Attempts to cause suppuration with this organism by subcutaneous injections failed. The second inoculations in two of the animals were ineffective when made intraperitoneally, the animals recovering although not so promptly as after the first injection. On the other hand, a second intraperitoneal injection proved fatal in all of three guinea-pigs tested. One animal received three injections, recovering promptly after the first which was given subcutaneously, more slowly after the second which was given intraperitoneally, but died from acute peritonitis and an overwhelming bacteremia after the second intraperitoneal injection. The time between the first and second injections in these animals varied from 4 to 24 days. On the whole the guinea-pigs proved more resistant than rabbits, none developing endocarditis or pericarditis.

Two dogs were injected, one into the heart and the other intravenously three weeks after the organism had lost part of the special characteristics. The one injected into the heart lost weight and was ill for 10 days, then seemed to get better, and when chloroformed a healing tricuspid and mitral endocarditis was found. The cultures made from the blood were sterile. In the other dog the injection seemed to have no effect.

The special affinity of this organism for the endocardium and pericardium is shown in the rabbit by the high percentage of involvement of these structures independently of the place of inoculation. The tendency in all cases of the endocarditis was to heal; none of the animals died spontaneously from this cause. In one instance of a beginning mitral endocarditis microscopic section of the nodule showed infiltration beneath the layers of the normal endothelium on both sides of the cusps.

Two months after the organism was isolated and when the peculiar properties had been lost, attempts to produce endocarditis and other lesions in rabbits failed. The apparent increased sensitiveness of the guinea-pig and rabbit, the result of relatively small, primary, and quite harmless doses of this organism, merits further mention. A study of the peritoneal smears in these animals showed that after the first inoculation prompt migration and phagocytosis of the cocci took place. Later many endothelial cells appeared which were markedly phagocytic for cocci, and leucocytes, especially those containing cocci, were taken up by the endothelial cells. After the second injection prompt migration of leucocytes and phagocytosis again occurred, which in no way differed from those after the first injection, but instead of the appearance of endothelial cells, phagocytic for leucocytes and cocci,

TABLE 4.  
SUMMARY OF EXPERIMENTS WITH STAPHYLOCOCCUS 334.

Animal	Days Since Isolation of Staphylococcus	Inoculation	Postmortem	Bacteriologic Examination	Remarks
Rabbit 9	9	3/1/08—6 c.c. broth culture into heart	3/24/08—Massive pericarditis; mural endocarditis	Coccus 334 isolated from pericardial endocarditic area, and heart's blood	Died from infection
"	9	3/1/08—5 c.c. broth culture intravenously	3/18/08—Tricuspid endocarditis	Coccus 334 isolated from heart and crushed portion of vegetation	Chloroformed
"	11	3/3/08—6 c.c. 24-hr. broth culture intraperitoneally	4/6/08—Pulmonary, aortic, and mural endocarditis; no peritonitis	Gram positive cocci in vegetation cultures. Blood sterile	Recovered promptly. Chloroformed
"	5	3/9/08—5 c.c. broth culture intravenously		Coccus 334 isolated from nodules on endocardium and heart's blood	Chloroformed
"	15	3/20/08—5 c.c. broth culture intravenously	3/25/08—Tricuspid and mural endocarditis	Coccus 334 isolated from heart's blood and nodules on tricuspid valve	Died from infection
"	15	3/20/08—15 c.c. 24-hr. broth culture intravenously	3/21/08—Tricuspid endocarditis (beginning)	Coccus 334 from heart's blood and pericardium	Died from second injection
"	8	2/28/08—3 c.c. 24-hr. broth culture intravenously	4/12/08—Massive pericarditis and pleuro-pericarditis; no peritonitis. Beginning mitral endocarditis	Coccus 334 from heart's blood and peritoneum	Died from second injection
"	10	3/14/08—5 c.c. broth culture intraperitoneally			
"	8	2/28/08—5 c.c. broth culture intraperitoneally	3/16/08—Fibrinous peritonitis; no endocarditis		
"	13	3/4/08—Intraperitoneally			
"	8	2/28/08—6 c.c. milk culture intraperitoneally	3/5/08—Acute sero-fibrinous peritonitis; no endocarditis or pericarditis		
"		3/3/08—8 c.c. broth culture intraperitoneally			
Guinea-Pig	5	2/25/08—Growth over surface of one agar slant in NaCl subcutaneously	3/26/08—No endocarditis; no peritonitis. Infarcts of spleen and kidneys		
"	12	3/4/08—5 c.c. broth culture intraperitoneally			
"	22	3/14/08—Same as above			
Dog	24	3/28/08—35 c.c. broth culture into heart	4/16/08—Healing tricuspid and mural endocarditis	Blood culture 2 days after injection gave coccus 334. Cultures after death sterile	Died from third injection Chloroformed. Sick for 10 days then improved

the exudate was thin with fewer leucocytes and few endothelial cells; phagocytosis was diminished and invasion of the blood took place without peritonitis in guinea-pigs, with peritonitis in rabbits. The organisms in the blood in one of the guinea-pigs were so numerous that there was no difficulty in finding them in smears. No evidence of phagocytosis was seen, notwithstanding that there were 15,000 leucocytes per c.mm. On concentration of the cocci by centrifugalization, then adding defibrinated blood and incubating for 15 minutes to three hours, phagocytosis failed to take place. On cultivation the coccus was susceptible to phagocytosis and a single injection into other guinea-pigs was without effect. Hence it would seem that the animals receiving more than one injection died from an acquired susceptibility and not from an increase of virulence of the organism. At the time the injections were made, the special characteristics had been lost, and this may explain the absence of endocarditis.

#### SUMMARY OF THE CASES OF PNEUMOCOCCUS ENDOCARDITIS.

Full protocols are given only of the cases that were studied closely, or in connection with which animal experiments were made. Clinically all the 14 cases in question were subacute in their course and while they must be regarded as cases of malignant endocarditis in that the patients all succumbed to the infection, they may be classed under the group designated by Osler<sup>1</sup> as chronic septic endocarditis. Four presented the picture of incompetent hearts from old lesions; the existence of an active endocarditis did not seem probable because there was no leucocytosis, practically no fever or other evidences such as petechial hemorrhages, infarcts, etc., to suggest the diagnosis of a more acute endocarditis. The blood culture was the means of making the diagnosis in three of the four cases, while the other was discovered at autopsy.

All but three of the 14 occurred between 15 and 29 years of age. The occupation of all was chiefly indoors. Six were male and eight female. In 10 cases the original source of the infection could not be determined; in two it seemed to be an attack of tonsillitis; in another the most probable source was pyorrhea alveolaris, while in still another it was an alveolar abscess. The exact duration of the disease could not be accurately determined but probably ranged from 4 to 12 months

<sup>1</sup> Osler, "Endocarditis," *Modern Medicine*, Philadelphia, 1908, 4, p. 133.

or more in the different cases. The blood cultures were the means of making an early positive diagnosis in almost all the cases. In some of the cases it was difficult to make a correct diagnosis because of the insidious onset, the chronicity, and most of all because the acute process was engrafted upon an old valvular lesion in seven cases; in five of these the old lesion was due to acute articular rheumatism; in three this point could not be definitely settled, while in four no definite previous heart lesion was present. Of the four last mentioned there was a definite source of infection in two while in the other two there was none. Petechial hemorrhages in the skin occurred late in nearly every one of the cases, long after the blood showed cocci. Probably the most constant feature was the development of a high grade of anemia of the secondary type. This was most pronounced in the cases which ran the more chronic course. A persistent leucocytosis was present in only four of the cases; the rest showed little or only temporary increase in the number of leucocytes. Definite chills occurred only rarely and in no case in which chills seemed to mark the onset of the disease. Early the fever was remittent. In some patients the temperature was practically normal for weeks and then showed an afternoon rise for a short period. Later, as embolism and petechial hemorrhage occurred, the fever took a septic or intermittent type associated with sweats. Definite joint symptoms were present in three of the cases, in none of which did suppuration occur. The relation between these cases and acute articular rheumatism is an interesting one. The coccus found resembles quite closely "*Micrococcus rheumaticus*" of Poynton and Payne, with, however, certain important differences. The facts that joint symptoms were few and mild in these cases; that arthritis developed only once in the animals injected, and that only five of the cases had had a previous acute articular rheumatism would seem to be proof that we are dealing with another form of infection in these cases. The valves on the left side alone were involved in 10 cases; the mitral valve alone seven times; mitral and aortic twice; aortic alone once. The right side alone was involved three times; tricuspid alone twice; pulmonary once. Both sides (aortic and tricuspid) were involved in one case. Autopsies were obtained in six of the cases. Anatomically the valvular lesions were all characterized by huge vegetative growths. The petechial hemorrhages were probably not all caused



by the breaking-loose of necrotic portions of the growths, but also by clumps of cocci too large to go through the capillaries. It should be noted here that necrotic areas on the vegetations were present at autopsy in the cases where other bacteria than the one responsible for the endocarditis were found with the latter in the blood post mortem. In two of the cases (311 and 292) the rapid failure near the end was undoubtedly due to the invasion of streptococci of high virulence. In the latter only hemolysing colonies were found and had not the coccus which was found during life also been found in cultures made from the vegetation after death, it might have led one to believe that these cocci had taken on the property of hemolysing blood as Buerger and Ryttenberg<sup>1</sup> claim to have found.

The infarcts did not show evidences of suppuration.

**Case 292.**—Girl, 21, single, student, family and previous history of no special significance. Always in good health; no history of tonsilitis or rheumatism; has not menstruated for the past four months, the flow having gradually grown less. Lost endurance and became nervous one year ago after a year's hard study. Improved somewhat during summer vacation and returned to work but had to give it up on account of weakness with some shortness of breath upon exertion; at the same time there was an increasing pallor with a sense of oppression in the chest, associated with a persistent cough, the result, it was then believed, of a severe cold. The temperature for one week at this time ranged between 99° and 102° F. Went south for the winter but failed to regain health; gradually grew worse and took to bed five weeks before death. The temperature now was 102° F.; there was a feeling of tightness and oppression over region of heart, a dry persistent cough, shortness of breath, great weakness and swelling of the abdomen. The blood showed 3,624,000 reds, 55 per cent hb., and 6,500 whites. No abnormal white or red cells were found. The urine remained normal until two weeks before death when it contained much albumin, epithelial, leucocytic, granular, and hyaline casts, and a few red cells.

*Examination (five weeks before death).*—Marked pallor of skin and mucous membranes; no icterus; no petechial hemorrhages. Throat and tonsils normal; lungs and pleurae normal. Heart enlarged 2 cm. beyond normal line, apex beat diffuse, loud systolic murmur over base with maximum intensity over pulmonic area; no thrill; pulse rapid, quick, easily compressible, of normal volume. The abdomen showed a shifting line of dulness; a mass in the left hypochondrium resembling spleen and somewhat tender, extending to crest of ilium below and to umbilicus on the right. Liver reaches two fingers' breadth below costal arch, left lobe tender. Rectum normal. Tuberculo-opsonic index 0.7; pneumococco-opsonic index 0.6; streptococco-opsonic index 0.7.

The presence of marked anemia of the secondary type with no leucocytosis and with enlarged spleen and liver pointed to Banti's disease. A possible hyperplastic tuberculous peritonitis with a greatly thickened omentum was also considered and the discovery of small and large moist rales over the right apex a short time previously

<sup>1</sup> *Jour. Infect. Dis.*, 1907, 4, p. 600.

supported this possible diagnosis. Blood cultures (suggested by Dr. Billings) gave a pneumococcus with certain peculiarities to be described, and a diagnosis of acute endocarditis was now made.

The patient grew worse slowly until four days before death when she had a pulmonary hemorrhage; she now failed rapidly from a seemingly overwhelming infection.

*Anatomic diagnosis.*—Acute vegetative and ulcerative endocarditis of the pulmonary valves and artery; subcutaneous and renal hemorrhages; multiple old and recent pulmonary infarcts; serofibrinous pleuritis; cloudy swelling of the heart, liver, and spleen; hyperplastic splenitis; anemia; passive hyperemia of the liver and spleen; hydrothorax; hydroperitoneum and hydropericardium; left fibrous pleuritis.

The body is well developed and medium sized. Pallor of the skin and mucous membranes; marked posterior lividity; rigor mortis. Irregularly distributed over the entire body are small, circumscribed, red spots from 1 to 3 mm. in diameter. The abdomen moderately distended, the cavity contains about two liters of slightly turbid fluid; lining smooth, no adhesions. The diaphragm reaches to upper border of sixth rib on left side and to sixth interspace on right side. The right pleural cavity contains a large amount of sero-fibrinous fluid; no adhesions. The left cavity contains a small amount of almost clear fluid and is partly obliterated by loose fibrous adhesions. The pericardium is smooth and contains 100 c.c. of serous fluid. The tongue normal. The uvula is slightly enlarged and edematous. The tonsils are enlarged. The tracheal lining is reddish. Both lungs are heavy; on the pleural surface of the right lung are several areas varying in size up to that of a small apple, somewhat raised, hard to the touch, and dark red in color; on section they extend as wedges toward the center of the lung and form sharp edges. The left lung has only three such areas, all of about the same size, i. e., 1 to 2 cm. in greatest diameter. The one in the left upper lobe shows a cavity produced by softening opening into a bronchus of the second degree. Over the infarcts the pleura is covered with fibrinous exudate. Both lungs contain much frothy, watery, mucoid fluid. The tracheo-bronchial lymph nodes are normal. The heart is about normal in size; the muscle pale with a yellow tinge; aortic, mitral, and tricuspid valves normal.

Pulmonary valves are covered with large grayish-yellow cauliflower-like vegetations, which nearly fill the lumen and at the base of which valves are ulcerated. Some of these excrescences are 2 cm. long. The intima of the pulmonary artery just above the valve also is ulcerated. At the point of bifurcation of the artery is a thrombus 0.75 cm. thick, solidly attached and readily followed to the periphery of the left upper lobe where it ends in the softened infarct just described. Several other small branches in the left lung are thrombosed. In the right lung is also extensive thrombosis and the branch leading to the largest infarct which is in the lower lobe is plugged throughout. On removing the thrombi from some of the smaller branches of the pulmonary artery the intima appears rough, even ulcerated.

The spleen is  $27 \times 14 \times 7$  cm. in size, capsule smooth, consistency somewhat uneven, dark blood on section. The liver shows a pale, yellowish parenchyma with indistinct markings and numerous pin-head ecchymoses. Gall-bladder normal. Also pancreas and adrenals. The kidneys are normal in size; capsule strips easily, leaving a smooth pale surface. The substance is soft. Genital organs normal.

*Bacteriological examination.*—The peritoneal, pleural, and pericardial fluids and heart's blood, plated on blood agar, yield a large number of grayish colonies surrounded, at the end of 24 hours, by a wide hemolytic zone, from 3 to 5 mm. in width depending

on the number of colonies present in the plate. Two drops of the material (including the blood) yield a countless number of colonies. No acid in inulin broth and the morphology and staining reactions are those of *Strept. pyogenes* and 2.5 c.c. of a 24-hour broth culture of this organism obtained from the blood killed a rabbit weighing 1,350 grams in 48 hours. Pure culture of streptococcus was obtained also from the spleen, liver, pancreas, heart, and from the recent, large area of infarction. In the softened infarct the staphylococcus aureus was found also. The bile contained the colon bacillus. Emulsions from vegetation on the tricuspid valve and the right ventricle contained both streptococci and pneumococci, the former outnumbering the latter 50 fold upon blood-agar plates. The pneumococcus-like colonies tended to adhere to the surface of blood agar, fermented inulin slowly, and at first the virulence was slight, a rabbit weighing 1,250 grams succumbing at the end of five days with a serofibrinous peritonitis and bacteremia after injection of a huge dose. No endocarditis was found. This organism showed capsules when first isolated. The strains obtained after death as well as one isolated from the blood before death were susceptible to phagocytosis. Plates from broth cultures of a strain obtained during life gave smaller and larger colonies of Gram positive diplococci and subcultures of the large colonies produced only large colonies and those of the smaller colonies yielded only small colonies. Three other strains of pneumococci obtained from protracted pneumococcal endocarditis all showed this peculiarity; strains from four cases of acute pneumococcal endocarditis failed to show it, the colonies being all large, and produced more green than the ones isolated from the chronic cases. Similar results are obtained at times with pneumococci obtained from the blood in pneumonia after long cultivation on the same artificial medium and after the strain begins to lose its vigor.

**Case 293.**—Clerk, age 21, admitted April 23, 1907, into the service of Dr. Billings at the Presbyterian Hospital. Had an attack of acute articular rheumatism three years ago. At this time he began to have heart trouble and ever since had shortness of breath on exertion, associated with palpitation of the heart and precordial pain. Denied venereal infection. Five months previously the patient had severe headache for a few days; was weak and had no appetite. A month later he began to have a dull pain in the palm of the left hand and later pain appeared in the region of the left hip. The pain in the hip lasted for a few days and then went away. Has had some pain but no swelling in left knee and ankle from time to time. During this time his general health was poor, no appetite, sleeplessness, nervousness, lost 30 pounds in weight. Two weeks previously developed severe pain in the left hip associated with a pulsating swelling above and behind the trochanter.

Physical examination on admission shows nothing of note except as regards the vascular system: Apex beat 5 interspace just inside nipple line, no thrill; dullness extends from 1 cm. to left of mammary line to right sternal border, above to third rib. A very distinct and loud diastolic murmur is heard best over aortic area and along sternum downward but not transmitted along vessels of neck and heard only faintly at apex. Capillary pulse present in fingers. Regular water-hammer pulse.

The spleen descends 2 cm. below costal margin; not tender, rather firm.

Over the anterior part of the left hip and a little behind the great trochanter may be seen and felt a pulsating heaving mass. On palpation a distinct thrill is felt; the pulsation is synchronous with the heart beat. The mass is distinctly tender, not very firm, and over it is heard a distinct bruit synchronous with pulse. Pulse in both popliteal arteries equal in volume and force. X-ray picture shows nothing abnormal.

On admission a blood count gave red corpuscles 4,336,000; leucocytes 6,900; hemoglobin 71 per cent. Differential white count: Small mononuclears, 10.8 per cent; large mononuclears, 4.3 per cent; polymorphonuclear neutrophils, 85 per cent; no eosinophiles or myelocytes.

April 29, 1907: Mass in left hip is much more extensive and more boggy to the touch. It is now difficult to get any pulsation and bruit is heard only indistinctly.

May 12, 1907: Needle introduced into aneurysm brings pure bright red blood, under strong pressure; and cultures from this gave the same organism as obtained from the blood previously.

June 16, 1907: Small pulsating mass as large as walnut under the left jaw; tender, rather soft; no distinct fluctuation, no redness.

June 24, 1907: A small petechial spot on right side near costal margin; another in the right conjunctiva. The hemoglobin 50 per cent.

July 19, 1907: Suddenly developed right hemiplegia and coma. Cannot move lower part of right face; wrinkles forehead nearly equally; closes and opens eyes. Eyes turned to left but can roll them to right. Cannot protrude tongue; right arm and leg absolutely immovable. Moves left arm and leg. Breathing stertorous. Heart condition not changed. Death.

All of four blood cultures, obtained in the usual manner, and repeated cultures from puncture of the aneurysm gave pure growths of a variably sized Gram positive diplococcus which in the early cultures in broth and milk formed short chains and clumps. Upon continued cultivation all of the strains have lost the tendency to grow in clumps and now grow in typical diplococcus form; the strains isolated first lost this property more rapidly than the ones isolated later in the course of the disease. The organism grew in large clumps in the fibrin clot, adhered moderately to the surface of the blood-agar slants when first isolated, and produced small colonies in blood-agar plates with only a slight greenish zone about them. No hemolysis occurred when normal or homologous blood was used in the plates. Two strains tested fermented inulin slowly, a property which they soon lost upon cultivation. The organisms were susceptible to phagocytosis in normal and homologous blood soon after isolation. The injection of rabbits showed that this was associated with a very low grade of virulence, both animals, each of which received 5 c.c. of a 24-hour broth culture intraperitoneally, recovering promptly. Here may be noted that the smears from the blood in the aneurysm showed a large number of diplococci and leucocytes but no definite evidence of phagocytosis. The size of the organism and the character of the colonies produced on blood agar resembled closely those sometimes produced by pneumococci which have been isolated from the blood in pneumonia and which have lost their vigor from long cultivation on artificial media.

**Case 311.**—F. S., girl 18 years old. Had always been in good health except for an attack of acute articular rheumatism six years ago, in which she developed mitral lesions. Admitted to the Presbyterian Hospital, October 28, 1907, under Dr. Sippy, to whom I am indebted for the opportunity of studying the case. Thanks are also due Dr. Pickering for the opportunity of studying the case after the patient left the hospital. Three months before admission the patient had an attack of severe sticking pain in the right chest, made worse by deep breathing and coughing. She had fever but no cough, no hematemesis. She remained in bed for a week, but never regained her former health and had a continued evening temperature of about 101° F. Three weeks before admission the patient noticed that her left ankle was swollen but not



painful. The swelling came on gradually and left in a week. No other joints have been swollen. On admission she complains of chilliness at times, prostration, and malaise. There has never been cough, localized discoloration of the skin, bloody urination, or trouble with the eyes. Bowels are regular; appetite good; sleeps fairly well.

*Examination.*—Decided pallor; no discolorations of the skin; fairly well nourished; the tonsils are not enlarged and have a normal appearance. Cardiac dullness is not increased to the right but extends just outside of mammillary line to the left. Auscultation reveals a presystolic and systolic murmur with maximum intensity over mitral area and transmitted slightly to the left. Lungs and pleural cavities normal. Spleen not palpable. Capillary pulse absent; pulse regular; easily compressible.

November 4, 1907: Examination for first time reveals a diastolic murmur heard best over aortic area and transmitted downward; also a capillary pulse in the finger nails.

November 18: Spleen distinctly palpable for first time.

November 25: Patient complains of severe sticking pain in left side and shoulder, made worse on deep breathing.

December 2: Sudden attack of severe pain in left hypochondrium under costal arch, associated with marked tenderness over spleen. Coagulation time of blood 3.5 min.

December 25: Suffering from an acute attack of pneumococcus tonsillitis. Very tender, swollen submaxillary gland; this disappeared without suppuration. Petechial hemorrhages occurred for the first time.

From this time on the patient failed rapidly, the opsonic index going far below normal and the leucocytosis going higher. A progressive anemia of the secondary type was now marked. The hemoglobin went to 38 per cent before death. The pulse with the patient in bed remained relatively low. It was always regular and showed no evidence of myocardial degeneration, until shortly before death when dilatation and arrhythmia of the heart occurred. Died January 22, 1908.

*Anatomic diagnosis.*—Ulcerative mitral, aortic, and mural endocarditis; multiple, infarcts in the spleen; petechial hemorrhages in the skin; anemia; fibrinous pleuritis and peritonitis; acute tracheo-bronchial lymphadenitis; hyperplasia of the spleen; edema of the lungs; cloudy swelling of the liver, kidneys, and myocardium; hypertrophy of the heart; obliterative fibrous pericarditis; fibrous pleuritis.

The body is that of a well-developed girl; 170 centimeters long. Over the face, forearms, trunk, and buttocks are numerous small reddish, slightly elevated spots measuring 1 to 3 mm. in diameter. Rigor mortis is present and posterior lividity well marked. The skin and mucous membrane of the mouth pale; the abdomen flat. The peritoneal cavity contains about a liter of slightly turbid fluid. The appendix normal. The right pleural cavity contains a small amount of blood-tinged, slightly turbid fluid and is partially obliterated by fibrous adhesions. The left pleural cavity is practically obliterated by fibrous adhesions and contains a small amount of blood-tinged fluid. The pericardial cavity is obliterated by fibrous adhesions. The thyroid gland and larynx are unchanged. The trachea contains a large amount of bloody frothy fluid. The tracheo-bronchial lymph glands are moderately enlarged, soft, and of a grayish-red color on cut surface. On the left lung pleura is rough and mottled grayish red. The upper lobe crepitates freely throughout; the lower lobe feebly. The cut surface exudes a large amount of bloody, frothy fluid. A piece cut from the lower lobe floats on water. The right lung presents similar alterations.



The heart measures  $15 \times 12 \times 4$  cm. and is much enlarged. The aortic and pulmonary valves are competent to the water test. The mitral leaflets are shrunken and difficult to make out. The whole margin of the mitral valve is the seat of vegetative growths. At one portion anteriorly the vegetations have grown over the mural endocardium of the left auricle. The tricuspid and pulmonary valves are unchanged. The aortic valve has on each leaflet an abundant mass, largely composed of clotted blood, and measuring  $5 \times 10$  mm. The vegetations have grown from the line of closure forward and at no point is the free margin implicated. The myocardium is grayish red. The wall of the left ventricle measures 18 mm. in thickness and that of the right ventricle 5 mm.

The spleen measures  $15 \times 7 \times 5$  cm.; capsule, smooth except over the regions about to be described. There are seen externally eight areas, grayish in color and measuring 5 cm. in their longest diameter; they are all depressed and contain a grayish-white center; surrounded by a zone of red. On section they are wedge-shaped and grayish white in color, not softened, surrounded by hyperemic splenic tissue.

The tonsils are small; the tongue, esophagus, mesenteric glands, intestines, and stomach are unchanged. The liver is grayish red in color and the lobular markings are distinct. The central veins are dilated. The gall-bladder contains thick black bile. The pancreas is normal. The kidneys, the uterus, ovaries, and tubes are normal.

*Microscopic examination.*—In the lungs are areas in which the alveoli are filled with blood and leucocytes. The colloid is increased and the cellular elements decreased in the thyroid. The lymph glands are hyperemic. The myocardium shows a moderate amount of fatty infiltration. There is hyperemia in the spleen and anemic infarcts with a hemorrhagic zone at the border. In the liver there are marked congestion and slight fatty changes. In the pancreas are seen areas of connective tissue proliferation between the lobules. There is marked hyperemia and connective tissue proliferation in the kidneys. There are no noteworthy changes in the ovary.

*Bacteriologic examination.*—The bile, serum, blood, peritoneal fluid, right pleural exudate, and vegetations were examined. In cover-glass preparations the bile is sterile; in the serum, blood, peritoneal fluid, right pleural exudate, and emulsion of the vegetations, are seen Gram positive cocci arranged in clusters and chains and in twos, and in the vegetation emulsion is a short Gram negative bacillus. Cultures prove the bile sterile and give a proportionately large number of pneumococci and a few staphylococci (aureus) from serum, blood, peritoneal fluid, right pleural exudate, and vegetation emulsion; also the streptococcus pyogenes from the blood, pleural and peritoneal fluids, and vegetation emulsion; bacillus mucosus from the vegetation emulsion.

*Animal experiments.*—Two rabbits injected with the pneumococcus 311<sup>4x</sup> developed endocardial lesions; in one the tendency seemed to be toward healing; the other died with ulceration of the aorta just beyond the cusps associated with multiple infarcts of kidneys and lungs. The blood cultures of the former were sterile, while in the latter they gave a coccus of the same characteristics as the one inoculated. A third animal died five days afterward without lesions and the coccus isolated from the blood had lost its special characteristics, behaving exactly as ordinary pneumococci. Four rabbits recovered without lesions after inoculation with large doses. The reason for the high percentage of recoveries with this strain is probably a low grade of virulence at the time of inoculation (90 days after isolation) in spite of the fact that the special characteristics were still retained. The virulence could not be increased at this time.

*Characteristics of the coccus.*—The characteristics of the organism isolated from the blood before and after death may be summarized as follows: It grows in colonies in the fibrin clot in the blood cultures, the liquid portion remaining clear for 48 hours. This characteristic I have not observed in pneumococci isolated from the blood in lobar pneumonia. In plain and dextrose broth the endocarditis organism produces a flocculent sediment consisting of large masses of closely adherent bacteria. Some of the masses attach themselves so tightly to the side of the test-tube that vigorous shaking fails to dislodge them. The broth remains perfectly clear. The strain isolated in the third blood culture lost this adhesive property on injection into rabbit after four months of artificial cultivation on blood agar, and cultures from the heart's blood grew like pneumococci from the blood in pneumonia; at the same time the strain assumed a moderate virulence and a corresponding lessened susceptibility to phagocytosis. The strain isolated in the tenth blood culture two months later still possesses this interesting characteristic to a moderate extent, now for over nine months since. Injections into animals of this strain have thus far failed to bring about any change.

In blood-agar plates the organism produces with a few exceptions small but somewhat variably sized colonies with a greenish zone resembling the pneumococcus except in that the production of green is not so marked. At no time was there noted hemolysis either of homologous or heterologous blood. In four of six cultures the blood-agar plates showed two varieties of colonies, a larger variety with a green zone and a smaller with no change in the media. Both consisted of Gram positive diplococci. Subcultures from the larger colonies always produced growths exactly like the original. The smaller colonies usually produced the smaller colonies, but sometimes the larger colonies also. At first it was thought that possibly we were dealing with a mixed infection but the fact that both varieties were Gram positive diplococci giving the same reactions and that the smaller variety sometimes produced the larger colonies led to the conclusion that they were the same organism, the ones producing the smaller colonies being less vigorous. Analogous observations have been made with respect to strains of pneumococci isolated in pneumonia and after cultivation on the same media for a long time. Upon blood-agar slants this particular strain had a marked tendency to adhere to the surface so that it would be necessary to tear the surface of the media to dislodge them. The bacteria composing the colonies were so adherent that an emulsion for opsonic work could be prepared only with great difficulty. In the water of condensation there appeared clumps of bacteria while the fluid remained clear. This property was accentuated in the successive blood cultures and lasted longer in the tenth strain than in that obtained in the third blood culture. When first isolated the organism fermented inulin slowly, but soon lost this power. It was not dissolved by ox bile. A definite capsule was demonstrated when first isolated in three of the blood cultures, but in two blood cultures the capsule was absent.

**Case 341.**—Woman, 35, mother of three children; no history of acute rheumatism or other recent infection. Complains of shortness of breath and a growing weakness for past year which has become so severe that she had to give up her work; is no longer able to lie flat in bed on account of dyspnea. Frequently has feeling of fulness and sense of oppression over heart and pain when especially short of breath. Swelling of feet toward end of day; subsides during night. Dry, hacking cough, occasionally spits a little blood; sputum lumpy. Has had no chill but thinks she has more or less fever every day. Menstruation more frequent than usual but less. Marked pallor; cyanosis; fingers slightly clubbed; no petechiae; pulsation of pericardium, systolic

thrill; loud presystolic murmur at apex in axillary line; pulmonic second sound accented; left auricle dilated; lungs and pleural cavity normal. Liver edge two fingers below costal arch; no edema; hb. 80 per cent, leucocytes 14,000. Blood pressure 130. Temperature 100.5°. In hospital for two months; temp. never went above 100° and to that point only twice. The leucocyte count became normal; pulse practically normal when in bed but high when walking about. Four months later she returned. There was no marked change in the heart; general condition, however, worse in spite of better care. Hemoglobin now 70 per cent; leucocytes 8,900; temp. 99.8°. Four months later gradually worse. Blood culture 30 colonies per c.c. of pneumococcus with the special characteristics common to the other strains isolated from cases of endocarditis, adhering to the surface of blood agar, growing in clumps and short chains, but susceptible to phagocytosis and not virulent for animals. (Patient still living when last heard from.)

*Animal experiments.*—May 13, 1908: Large Belgian hare. 10 c.c. 24-hour blood-broth culture intravenously; 10 c.c. 24-hour blood-broth culture intraperitoneally and subcutaneously.

May 14: Seems ill. Blood cultures.

May 15: Average of 8,000 green colonies per c.c. of blood. Seems better.

May 16: Seems quite ill.

May 18: Found dead. Slight fibrous deposit at site of subcutaneous injection. No peritonitis. Fibrinous pericarditis; vegetative endocarditis of aortic and mitral valves; mural endocarditis of left ventricle and right auricle; the vegetation of the latter has grown down and become adherent to the mitral valve. Smears from the pericardium show large numbers of Gram positive diplococci many of which are within leucocytes in various stages of disintegration. 250 colonies per c.c. of blood on blood-agar plates. Cocci obtained by centrifugation resisted phagocytosis by normal human and homologous rabbit's leucocytes in homologous serum. Cultures from the pericardium, the vegetations, and the heart blood gave pure cultures of the coccus inoculated. After cultivation for two days the cocci were taken up quite freely by human leucocytes in human serum and rabbit's leucocytes in rabbit serum. Subsequent injections of large doses of the coccus in rabbit and guinea-pig produced death from pneumococemia, the coccus now having the characteristics of ordinary pneumococci.

May 11, 1908: Medium sized albino. 5 c.c. 24-hour milk culture intraperitoneally.

May 12: Seems perfectly well. Peritoneal fluid watery, free from bacteria; few leucocytes and endothelial cells.

May 15: Perfectly well.

May 16: Ill. Blood culture.

May 18: Well. Blood culture sterile.

May 19: Perfectly well.

May 20: Seems well; chloroformed. Several small thrombi in portal vein.

May 22: In cultures of blood growth of Gram positive diplococci which resemble the coccus inoculated; they adhere to the surface of the blood agar, are susceptible to phagocytosis in human serum by human leucocytes. The growth is less vigorous and the production of green about the colonies on blood-agar plates is less marked than originally.

**Case 353.**—Banker, 48, married; Dr. Clarke's patient. Inflammatory rheumatism 23 years ago. During 10 subsequent years one or two attacks annually. Joints

much inflamed during the attacks. Tonsilitis as a child. Six years ago an attack of tonsilitis and gripe. Pyorrhea for past four years. Bronchitis relatively frequent. One year ago pain over epigastrium, icterus, and clay-colored stools (once only). During last year much mental but little physical depression. Nearly a year ago dyspnea noticed on going up stairs, with pain over heart and, soon, attacks of angina pectoris. Fever and chills began nine months ago, temperature during the evening  $99\frac{1}{2}$  to  $103^{\circ}$ ; usually subnormal in morning. Repeated chills and chilly sensations for past four months. Weight reduced from 200 to 130 pounds. Universal petechiae noticed one week ago. Went to bed nine months ago and has been there most of the time since.

*Physical examination.*—Much emaciation, sallow and listless. Multiple petechiae—disappearing now—over entire body. Slight mitral murmur, systolic; heart dulness increased slightly to left. Gums spongy and teeth in poor condition, one upper incisor loose. Blood culture gives about 16 colonies to 1 c.c. blood on blood agar and potato. The injection of 50 million of the homologous coccus had no apparent effect upon the patient's condition. Death about 11 months after the definite symptoms appeared.

The coccus isolated from the blood produced typical green colonies upon blood-agar plates. It resembled in every way the colonies of pneumococci from the blood in pneumonia, except that on blood-agar plates as well as slants, the cocci grow tightly to the surface of the medium. This property was lost in one week. It also had the property of growing in clumps in the fibrin clot of the broth cultures and only slowly produced turbidity of the fluid portion of the broth. The early subcultures fermented inulin slowly when grown on litmus inulin agar; this power was promptly lost. The organisms were not dissolved by ox bile; capsules demonstrable by the Welch and Buerger method, and stained well by Gram's method. In milk and broth the coccus tended to grow in clumps of from 50 to 200, but there were also single and short chains of diplococci. The second subculture in broth was freely susceptible to phagocytosis in normal blood. This was also true of the coccus when grown on agar slants and suspended in salt solution.

*Animal experiments.*—Four rabbits inoculated with large doses of the first daughter culture in blood broth, 24 hours old. Rabbit 168 received 3 c.c. intravenously and 3 c.c. intraperitoneally, dying five days later, and typical encapsulated diplococci were isolated from the blood in fair numbers. Rabbit 169 was given 3.5 c.c. intraperitoneally and 3.5 c.c. subcutaneously; seemed perfectly well four days later, but died in 10 days from a pneumococcemia. A small subcutaneous abscess was found at the site of injection but no peritonitis, pericarditis, or endocarditis.

Rabbit 167, June 19, 1908: Injected 3 c.c. intravenously and 3 c.c. intraperitoneally and 3 c.c. subcutaneously.

June 20: Seems fairly well.

June 23: Seems to have recovered.

June 31: Seems fairly well but is losing weight.

July 19: Large abscess at point of subcutaneous injection but seems fairly well except labored breathing upon exertion or when handled. A sharp systolic murmur can be heard with the stethoscope over the heart.

July 20: Chloroformed. Massive adhesive and fibrinous pericarditis; vegetative endocarditis of mitral valve; subcutaneous abscess. No peritonitis. Smears from the crushed portion of the vegetation and from material from the pericardium show a



arge number of Gram positive diplococci while those from the heart's blood and peritoneal fluid are sterile. Cultures from the heart's blood, vegetations, peritoneal fluid, and pericardium on blood agar and in broth are sterile.

Rabbit 170, June 19, 1908: 3 c.c. intravenously.

June 20: Blood cultures show moderate number of pneumococci. Seems fairly well.

June 23: Is losing weight but otherwise seems fairly well.

June 29: Found dead.

Autopsy shows a huge fibrinous pericarditis and multiple abscesses of the liver which are situated about radicles of the portal vein and from which thrombi spring and extend backward. The endocardium is smooth. Smears from the pericardium and the thrombi show a large number of Gram positive diplococci some of which are within leucocytes. No evidence of phagocytosis in the blood. Cultures on blood-agar plates show typical green colonies.

**Case 359.**—Girl, 18 years old, under the care of Dr. Billings. Was always delicate; usual diseases of childhood; menses at 14; regular as a rule; frequent nose bleed since 12; worked hard at her studies last year, and became weak both physically and nervously. At present complains of weakness, anorexia, restless sleep, dyspnea after exertion, black spots before eyes on rising suddenly. Had tonsilitis six or seven months ago, was in hospital for a week, but felt well afterward. No history of rheumatism. Hemoglobin 50 per cent, reds 3,560,000; whites, 21,000. Urine normal. Pulse small, 140 per minute; respirations 30; temperature 101.6°. Diffuse apex beat; left border of heart 11 cm. from center of sternum; left base to the second rib and right border of cardiac dulness to the center of the sternum; very loud systolic rough murmur at the mitral and apex areas, transmitted to the axilla and back; over center of sternum a churning murmur with both actions of the heart; pulmonic second sound greatly accentuated and louder than the aortic which is also somewhat accentuated. Spleen palpable at costal arch. Liver not enlarged. Abdomen scaphoid. No purpuric spots; no glandular enlargements. Blood cultures gave 500 colonies of pneumococci per c.c. of blood, resembling those isolated from other cases of endocarditis. The cocci grew in the fibrin clot of the blood, were susceptible to phagocytosis, and had practically no virulence to animals. The colonies at first were tightly adherent to the surface of blood-agar slants and produced a viscid sediment in broth and no uniform turbidity. Both these properties were soon lost.

The patient was placed at absolute rest in bed but gradually grew worse and died two months later. Three times she was injected with approximately 75 million of dead pneumococci (own); an improvement followed each injection lasting 24 hours.

*Animal experiments.*—The injection of huge doses intravenously and intraperitoneally in three rabbits and one guinea-pig after the special characteristics were lost failed to produce endocarditis. One rabbit died of a pneumococcemia without peritonitis. The rest recovered promptly.

#### CHARACTERISTICS OF THE COCCI STUDIED.

The more important characteristics only are summarized here:

The cocci always produced more or less greenish discoloration around the colony upon blood-agar plates but never a zone of hemolysis. The variation in this respect was great; usually, however,



the strains from endocarditis produced less green than the strains from pneumonia.

Most of the strains soon after isolation fermented inulin to a mild degree, a property which all soon lost. Pneumococci from the sputum in pneumonia ferment inulin more rapidly than those isolated from the blood. They also retain this property longer than the latter when cultivated upon artificial media. Hence it would seem that the lessened fermentative powers of the endocarditis cocci might be looked upon as owing to a long residence in the blood.

When first isolated in broth they grew in the fibrin clot in large colonies, a form of growth I did not observe in the study of over 300 strains of pneumococci from cases of pneumonia. The endocarditis cocci grew in clumps in the broth and produced a diffuse turbidity slowly, and upon agar slants the colonies grew more or less tightly to the surface. The stained specimens gave diplococcus forms and chains. The chain formation as well as the property of growing in clumps in broth, and of adhering tightly to the surface of blood-agar slants, disappeared after cultivation for a variable period, depending upon the degree to which these special characteristics were present in the beginning. The more chronic the course of the infection and the later in the course of the disease the organism was isolated, the more marked and the longer these special characteristics lasted.

By cultivation on artificial media all the strains change gradually into typical lanceolate diplococci, often capsulated, growing as typical pneumococci in broth and on blood-agar slants. On animal inoculation this modification often occurred abruptly. It has been impossible to so modify strains of *Strept. viridans* which these organisms much resemble.

*Agglutination.*—The cocci used in the agglutination tests (see Table 5) were grown upon the surface of plain agar slants and suspended in salt solution. The results given were read 12 hours after the mixtures were made. The table shows a high degree of agglutinating power of the serum from the cases of endocarditis with respect to pneumococci isolated from endocarditis, a moderate power for two strains of pneumococci isolated from the sputum and blood of two cases of pneumonia, and no agglutinating power in the dilutions used, for *Strept. pyogenes*, *Strept. mucosus*, and *Strept. viridans*. Pneumonic

serum, obtained the day after crisis, in the dilutions used, had approximately the same agglutinating power over pneumococci isolated from pneumonia and endocarditis but none over the streptococci. Normal serum showed a slight agglutinating power for two strains of pneumococci isolated from endocarditis while for pneumococci from pneumonia and the streptococci it had no agglutinating power. Similar results were obtained with other sera and strains obtained from cases of endocarditis.

TABLE 5.

COMPARATIVE AGGLUTINATING POWER OF NORMAL, ENDOCARDITIC, AND PNEUMONIC SERA ON STREPTOCOCCI AND PNEUMOCOCCI.

ORGANISM	NORMAL SERUM		ENDOCARDITIC SERUM		PNEUMONIC SERUM		NORMAL SALT SOLUTION
	1-100	1-500	1-100	1-500	1-100	1-500	
<i>Strept. pyogenes</i> .....	o	o	o	o	o	o	o
<i>Strept. mucosus</i> .....	o	o	o	o	o	o	o
<i>Strept. viridans</i> .....	o	o	o	o	o	o	o
Pneumococcus from sputum in pneumonia.....	o	o	++	o	+	o	o
Pneumococcus from blood in pneumonia.....	o	o	++	o	+	o	o
Pneumococcus 311, from blood of patient with endocarditis.....	+	o	+++	++	+	o	o
Pneumococcus 292, from blood of patient with endocarditis.....	o	o	++	++	+	o	o
Pneumococcus 293, from blood of patient with endocarditis.....	+	o	+++	++	+	o	o

#### THE ENDOCARDITIS COCCI, MODIFIED PNEUMOCOCCI.

That we are not dealing with *Strept. pyogenes* in these cases, even though the early smears show chains, is certain for obvious reasons. That the strains should be looked on as belonging to the pneumococcus group and as having undergone environmental modification and not be regarded as strains of *Strept. viridans* seems warranted because all the strains have been changed into typical pneumococci by prolonged artificial cultivation or animal inoculation; because they fermented inulin when first isolated; because the serum from the cases had a high agglutinating effect for known typical pneumococci and not for either *Strept. viridans* or other streptococci; because the environmental modification grew more marked as the infection progressed, and because the opsonic power of the serum of the cases showed a decided specificality for the various endocarditis strains as well as for pneumococci from pneumonia, but not for streptococci.

RESULTS OF OBSERVATIONS ON OPSONIC INDEX, THERAPEUTIC INOCULATION, LEUCOCYTES, AND SERUM, IN THE CASES OF  
PNEUMOCOCCUS ENDOCARDITIS.

The observations recorded in Chart 2 were made upon case 311 (see page 259), a case of pneumococcus endocarditis of the mitral valve which began insidiously on top of an old endocarditis the result of rheumatism, and ran a course of six months or longer. The case offered an excellent opportunity for a study of the blood in various ways because the infection continued for a long time without marked variations. During three months this study was interrupted only three times by acute phenomena, twice on account of infarctions (as shown at the post mortem), and once by an attack of pneumococcus tonsillitis.

Repeated blood cultures were made to learn whether bacteraemia in endocarditis is constant and to determine the number of cocci in the blood; and also to learn whether the inoculation of dead bacteria would have any influence upon the number of cocci in the blood. Ten cultures were made; of these all but the first showed pure cultures of pneumococcus. Cultures in broth sometimes proved sterile, but upon agar plates from 35 to 50 colonies per c.c. of blood developed. There was a steady diminution in the number of cocci during the period of the first eight injections, but the patient showed no signs of improvement; instead he slowly grew worse. This diminution was probably only apparent owing to greater clumping of the bacteria as there was noted an increased agglutinating power of the serum in this case as time went on. There seemed to be no causal relation between the number of leucocytes, the opsonic index, and the number of bacteria in the blood. In the beginning the opsonic index was tested with respect to three strains of pneumococci, one from pneumonia, one from a similar case of endocarditis and the homologous strain. The results corresponded closely. The results given in Chart 2 were obtained with the strain from another case of endocarditis which was used because it was especially adaptable for the work. The Wright method as well as dilution method gave similar results.

The facts brought out by the study of the opsonic index, the leucocytes, and the temperature curve in this case may be summarized briefly as follows: The average opsonic index before the injections were

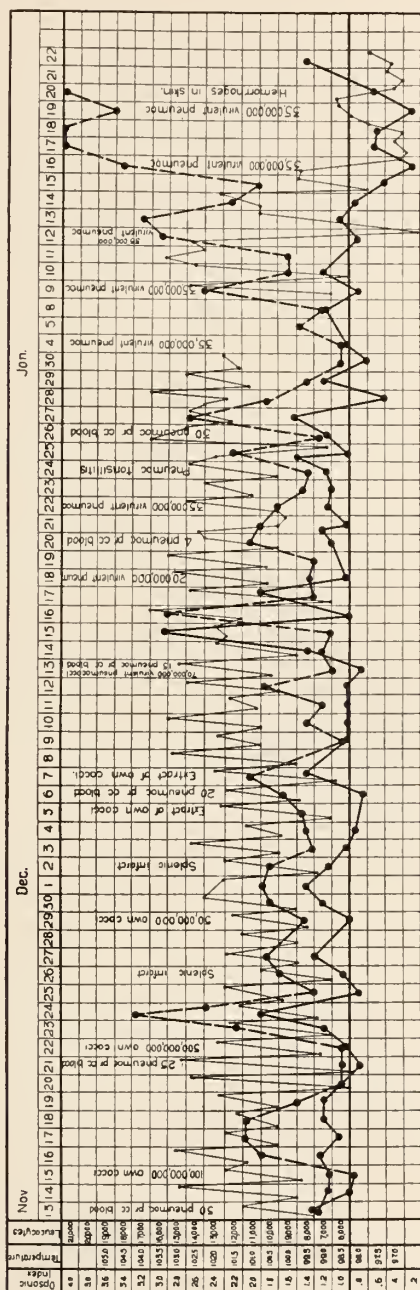


CHART 2.—Opsonic Index, Temperature, and Leucocyte Curves in Case 311. Solid heavy line = pneumococco-opsonic index; solid line = temperature; broken heavy line = leucocytes.

begun was above normal, hence a lack of opsonin could hardly be held responsible for the continuance of the infection. The injection of the homologous as well as of dead virulent pneumococci as indicated on the chart caused a rise in the opsonic index in nearly every instance. The rise was usually marked upon the day following, going still higher on the second day, and then usually in one or two days more the index dropped to a point lower than previous to the injection. A rebound sometimes occurred. A negative phase was not observed; if there was any it occurred inside of 24 hours. Dead virulent pneumococci were injected because it was thought that the homologous organism might have lost the power to stimulate properly the immunizing mechanism of the host. They produced a greater local and general reaction. The injection of 70 million on December 13 was more effectual in raising the opsonic index and leucocytosis than the injection of 500 million killed cocci of the patient's strain on November 2. The injection of extracts in salt solution of the homologous organisms upon two occasions was followed by a drop instead of a rise in the opsonic index.

Other things being equal, the rise in the leucocytes and in the opsonic index was roughly proportionate to the amount injected. Early the rise and fall in the opsonic index and leucocytes ran parallel, later, as the general condition of the patient became worse, the opsonic index falling, the reverse seems to have been true.

Previous to the time when the temperature became subnormal and the patient moribund there was a distinct or slight rise in temperature as the opsonic index and leucocytes rose after seven inoculations and a drop after three.

There was no marked change in the patient's general condition one way or the other until later, the opsonic index then being far below normal, when for 24 hours following the injection there was a rise in the opsonic index, and the patient seemed to be more comfortable, the pulse stronger, and the general condition better. That there may be a close relation between the temperature and the opsonic index is indicated by the observations on December 24 and 25. The index on these days was taken when the temperature was the highest at midnight, and lowest, four hours later. The results show a decided drop in the index with the drop in the temperature which was associated



with profuse perspiration. Hence great variations in the index may occur in short periods of time. We note further the agonal rise in the index, the tendency of the index to drop to a lower level after the temporary rise than it was previous to the injection, and the drop after the injection of the extracts.

From a consideration of all the facts it would seem that the injection of bacteria had no curative effect in this case. Opsonification and phagocytosis seem to have been of no importance in combating the infection in spite of the fact that we were dealing with a coccus which was susceptible to phagocytosis on cultivation and of low virulence to animals. Now a study of Table 6 shows that normal blood (washed

TABLE 6.  
THE COMPARATIVE PNEUMOCOCCIDAL VALUE OF NORMAL AND ENDOCARDITIS BLOOD.

MIXTURES *	PHAGOCYTOSIS 15 Min.	NO. OF COLONIES IN BLOOD-AGAR PLATES		
		At Once	24 hr.	48 hr.
1. Washed normal blood + normal serum + Pn. 311.....	3.	780	5	0
2. Washed normal blood + 311 serum + Pn. 311.....	3.4	550	25	0
3. Washed 311 blood + normal serum + Pn. 311.....	2.8	350	134	10
4. Washed 311 blood + 311 serum + Pn. 311.....	4.6	800	4,000	∞

\* Two sets of tubes were made, one for the study of phagocytosis, the other for the study of pneumococcal effect, each containing equal parts of serum and washed leucocytes and broth culture of the coccus. The washed normal blood contained 20,000 leucocytes per c.mm. and 311 blood 22,000. The colonies represent the bacteria per loop plated out at the time indicated. The experiment was performed on a day when the leucocyte count was 15,000.

∞ = uncountable.

normal leucocytes + serum) and the patient's washed leucocytes in normal serum had a marked destructive power over the coccus from the patient's blood, while the leucocytes in the patient's serum had no such power. This must be ascribed to loss of the power of the leucocyte in the presence of this particular serum of digesting the bacteria and not to the lack of phagocytosis. Other tests showed that the organism grew well in the patient's serum. The lack of destructive power of the patient's blood is not specific for the homologous coccus because other strains grew invariably in the patient's defibrinated blood, while in normal defibrinated blood, with the same number of leucocytes, there was destruction.

In a previous paper<sup>1</sup> I have shown that the leucocytes in croupous

<sup>1</sup> *Jour. Infect. Dis.*, 1906, 3, p. 683.

pneumonia and other acute infections with an active leucocytosis may have a higher phagocytic value for pneumococci than normal leucocytes. A study of the phagocytic value of the leucocytes in this case is shown in Table 7 which reveals that the opsonic index usually cor-

TABLE 7.  
THE COMPARATIVE PHAGOCYTIC POWER OF NORMAL AND ENDOCARDITIS (311) LEUCOCYTES.

MIXTURES*	PHAGOCYTOSIS IN 15 MINUTES							
	Nov. 22	Nov. 26	Nov. 30	Dec. 12	Dec. 25	Dec. 26	Dec. 27	Dec. 30
Washed normal blood + normal serum + Pn. 292.....	3.4	2.7	1.9	2.	2.4	4.5	3.	5.
Washed normal blood + 311 serum + Pn. 292.....	3.1	3.	2.4	2.4	3.9	5.9	3.4	4.
Washed 311 blood + normal serum + Pn. 292.....	3.5	4.3	2.	3.	4.7	3.5	2.8	5.5
Washed 311 blood + 311 serum + Pn. 292.....	3.	3.9	2.8	3.6	6.	3.	4.6	3.4
Leucocyte Count on Dates Indicated	6,500	10,000	8,500	11,000	12,500	7,500	15,000	6,800

\* The number of leucocytes was controlled by counts of the washed blood. Equal numbers of normal and patient's leucocytes were used. Differential leucocyte counts showed the proportion of polynuclears to be approximately the same in both. The strain used in these tests was obtained from another but similar case of endocarditis. It had been cultivated for a longer time and gave an even suspension. Controls with the patient's own organisms gave the same results.

responds when normal and patient's leucocytes are used. Two exceptions occur: November 26 and December 26. On these dates the index is high with normal leucocytes and low with patient's leucocytes; when the index is low with the former it is always low with the latter. The most significant point in this connection is that the patient's leucocytes are less active or equally as active as normal leucocytes on the date when there is no leucocytosis and more active when there is leucocytosis. This is true usually both with normal and patient's serum. In this case there is, then, not only a difference in the phagocytic activity of leucocytes upon different dates but, what is more significant, a lack in the power of destroying the engulfed bacteria. The increased phagocytic activity during leucocytosis is not associated with an increased destructive power.

Chart 3 gives a summary of the observations on Case 293. The chart shows that the injection of the homologous dead organisms was followed by a rise in the leucocytes; the injection of a smaller dose caused no marked changes on one occasion, a pronounced increase in the opsonic index in another, while a marked decrease followed the third injection which contained a large dose. The temperature for

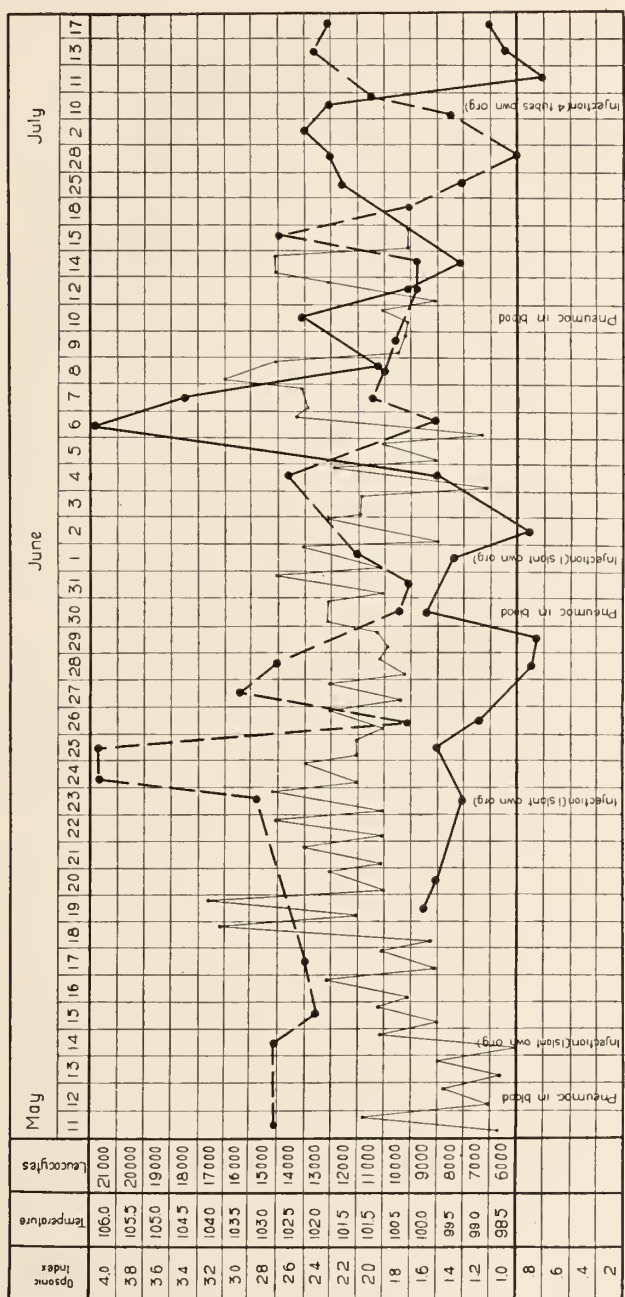


CHART 3.—Opsonic Index, Temperature, and Leucocyte Curves in Case 293. Solid heavy line = pneumococco-opsonic index; solid fine line = temperature; broken heavy line = leucocytes.

several days after the injections showed a moderate drop after two, a definite rise after one, and no apparent change after the other injection. There was, therefore, no constant relation between the opsonic index, the leucocytes, and the temperature following the injections in this case. The exceptionally high opsonic index of 4.0 upon June 6 may have been owing to autoinoculation from the submaxillary aneurysm rather than the result of the injection of bacteria.

Table 8 shows that the patient's leucocytes were more active than normal leucocytes toward the strain isolated in this case as well as

TABLE 8.  
INCREASED PHAGOCYTIC POWER OF PATIENT'S (293) LEUCOCYTES AS COMPARED WITH  
NORMAL LEUCOCYTES.

MIXTURES	PHAGOCYTOSIS IN 15 MINUTES	
	Normal (7,000 Leucocytes per c.mm.)	293 (7,500 Leucocytes per c.mm.)
Normal serum + Pn. 293.....	6.	9.
Normal serum + Pn. 245.....	5.5	11.4
Serum 293 + Pn. 293.....	5.1	8.7
Serum 293 + Pn. 245.....	4.7	9.

toward pneumococcus from the sputum of a case of pneumonia. The patient's leucocyte count on the day of the experiment was 12,000. The homologous organism grew readily in the patient's serum which was agglutinating in dilutions of 1 to 1,000, but grown in the patient's serum for 24 hours the strain showed a marked diminution in susceptibility to phagocytosis, especially in the patient's blood.

Chart 4 is a summary of the more important observations on another case (292) of pneumococcal endocarditis of the pulmonary valve in which no source of infection could be found and in which no previous heart lesion was present. The duration probably was over a year and death seemed to be caused by a terminal streptococcemia. The injection of 2,500,000 dead homologous cocci was followed by a rise in the opsonic index from 0.55 to 1.1 in two days. The great drop in temperature and pulse shown in the chart at this time may have been due to a large dose of morphine given, because the second injection which also raised the opsonic index was not associated with such drop. There was decided symptomatic improvement in the patient's condition for two days following the first injection. There was no such marked change after the second injection, probably because of the

invasion of streptococci which may have been the immediate cause of death. On the other hand the leucocytes, temperature, and pulse rapidly went up while the opsonic index went down. The streptococco-opsonic index at the time of death was also very low, whereas it had been nearly normal.

Table 9 gives the results of a study of the serum and leucocytes of case 353. Pneumococcus 353 is the coccus isolated from this case,

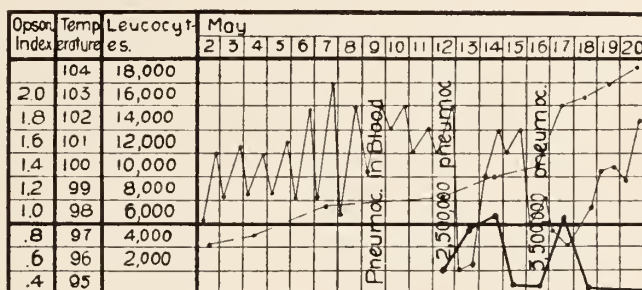


CHART 4.—Opsonic Index, Temperature, and Leucocyte Curves in Case 292. Solid heavy line = pneumococco-opsonic index; solid fine line = temperature; broken fine line = leucocytes.

TABLE 9.

THE PHAGOCYTIC VALUE OF THE LEUCOCYTES AND OPSONIC POWER OF THE SERUM FROM A CASE OF ENDOCARDITIS (353).

Mixtures	Agglutination	Phagocytosis in 15 Min.
Normal blood + normal serum + Pn. 353.....	No Agglutination	5.
" " + patient's " + Pn. 353.....	Marked "	0.2
Patient's " + normal pooled serum + Pn. 353.....	No "	2.4
Patient's " + patient's serum + Pn. 353.....	Marked "	2.
Normal blood + normal pooled serum + Pn. 291.....	No "	4.85
" " + patient's serum + Pn. 291.....	" "	4.5
Patient's " + normal pooled serum + Pn. 291.....	" "	4.3
" " + patient's serum + Pn. 291.....	" "	5.

while pneumococcus 291 is an ordinary pneumococcus isolated weeks previously from the blood of a case of lobar pneumonia. The blood was obtained five days before death and two days after the injection of 50 million heated homologous cocci. The opsonic index for the homologous cocci is 1.2 when obtained with washed normal blood, and 0.9 for strain 291. On the other hand when the patient's leucocytes are used the total amount of phagocytosis is less than half as great and the opsonic index is .8 for the homologous coccus and 1.1 for strain 291. The patient's leucocytes are as active phagocytically as normal leucocytes for strain 291 (see Table 5). The difference is not



the result of agglutination because there is the same amount of agglutination when normal and patient's leucocytes are used in the presence of patient's serum and there is no agglutination in the presence of normal serum. Further, the patient's serum was strongly agglutinative of pneumococcus isolated from endocarditis for which the leucocytes were as active phagocytically as normal leucocytes, the opsonic power of the patient's serum in both instances being high. It would seem therefore that the diminished phagocytic activity of the patient's leucocytes is closely related to the homologous organism and that the patient's serum is less opsonic than normal serum. There is then in this case a specific diminution of the phagocytic power of the patient's blood with respect to the organism causing the infection. Table 10

TABLE 10.  
COMPARISON OF GROWTH OF PNEUMOCOCCUS 353 IN SERUM 353 AND NORMAL SERUM.

MIXTURES	AGGLUTINATION	NUMBER OF COLONIES IN BLOOD-AGAR PLATES	
		At Once	20 Hours
Normal serum + Pn. 353.....	No agglutination	4,300	3,000
Patient's " + ".....	Marked "	4,200	7,500
Normal " + " 31I.....	No "	5,800	3,100
Patient's " + ".....	" "	6,500	8,900

shows that the pneumococcus isolated from the patient, as well as a strain from another case of endocarditis, grows more readily in the patient's serum than in normal serum, there being more than twice the number of colonies at the end of 24 hours in the patient's serum in spite of the marked agglutination.

#### SUMMARY OF ANIMAL EXPERIMENTS WITH PNEUMOCOCCI.

Table 11 gives a summary of the animal experiments with pneumococci. It shows that endocarditis developed in five of ten animals inoculated before the special characteristics of the pneumococci were lost. In none of these were the valves injured before inoculation. Pericarditis developed in six cases; in two, endocardial lesions were absent while pericarditis developed and also beginning thrombosis of the portal vein. The apparent affinity of these organisms for the endocardium and pericardium is quite apparent. Pericarditis developed quite independently of the place of inoculation. The right heart was involved only once; in the other cases the mitral was affected

TABLE II.  
SUMMARY OF ANIMAL EXPERIMENTS WITH PNEUMOCOCCI FROM ENDOCARDITIS.

Animal	Organism Inoculated	Days Cultivated Since Isolated	Special Characteristics	How Inoculated	Postmortem Findings	Bacteriologic Findings	Remarks*
Rabbit	311ix	90	+	5 c.c. milk culture intravenously	Healing aortic, pulmonary, and mural endocarditis; localized pericarditis	Smears from endocardium and pericardium show Gram positive diplococci	Chloroformed 21 days after infection. Seemed quite well
"	311ix	60	+	15 c.c. intravenously 24 hr. blood-broth culture	Ulcerative aortitis; infarcts of kidney and lungs; pleuro-pericarditis	Organism recovered	Death in 28 days from inoculation
"	311ix	90	+	5 c.c. blood-broth culture	No localization, no peritonitis	Smears and cultures yield typical lanceolate diplococci which have lost their special characteristics	Death in 5 days from pneumococcemia
"	341ix	3	+	10 c.c.† intravenously; 10 c.c. intravenously and subcutaneously	Vegetative endocarditis of aortic and mitral valves; endocarditis left auricle and left ventricle. Fibrous pericarditis; no peritonitis	Coccus of same characteristics isolated from blood pericardium, vegetations	Death in 11 days after inoculation
Guinea-Pig	341	2	±	3 c.c. into heart 3 c.c. intraperitoneally	Death without lesions in 48 hr.	Pneumococci isolated like those from blood of pneumonia	Organism when inoculated had lost part of former characteristics
Rabbit	341	3	+	5 c.c. intravenously 4 c.c. into heart	Death in 48 hours from peritonitis and pneumococci	Pneumococci typical of those from blood of pneumonia	
"	353	2	+	3 c.c. into peritoneum 3 c.c. intravenously 3 c.c. intraperitoneally 3 c.c. subcutaneously	Massive, adhesive, and fibrinous pericarditis; vegetative endocarditis of mitral valve; subcutaneous abscesses; no peritonitis	Smears from vegetations and pericardium show abundance of Gram positive diplococci	x
"	353	2	+	3 c.c. intravenously Injection of 5 c.c. organism 311ix 4 weeks previous	Pericarditis; portal thrombosis; no endocarditis	Pure cultures of pneumococci from heart's blood, epicardium and portal vein	Death in 48 hours
"	353	2	+	3 c.c. intravenously	No anatomic lesions	Typical encapsulated diplococci	
"	353	2	+	3-5 c.c. intraperitoneally	Died in 48 hours	Many encapsulated pneumococci in blood	
"	359	7	0	10 c.c. subcutaneously	Chloroformed 2½ months later. No lesions	Blood cultures sterile	Loss in weight for a time, then recovery
"	359	7	0	7 c.c. intraperitoneally	do	do	
"	359	7	0	10 c.c. intraperitoneally	do	do	
"	359	7	0	7 c.c. intravenously	do	do	
Guinea-Pig	350	7	0	7 c.c. intraperitoneally 4 c.c. intraperitoneally 4 c.c. into heart with injury to valves	Fibrinous pericarditis; beginning endocarditis; thrombosis of portal vein; osteomyelitis	Cultures from heart's blood, pericardium, and thrombus	Animal died 18 days after injection
Rabbit	344	2	+	10 c.c. intravenously 10 c.c. intraperitoneally; 10 c.c. subcutaneously	Serofibrinous peritonitis	Large number of pneumococci in the blood; capsules present	Death 5 days after inoculation
"	292	10	0	10 u. intraperitoneally			

\* Only the animals showing the more important lesions are here tabulated. A number which showed a healing endocarditis are not included.

† The number of c.c. of the culture means a 24-hour culture in broth unless otherwise stated.

three times and the aortic twice. The failure to produce endocarditis after the special characteristics of the pneumococci were lost is interesting; of 16 rabbits injected with various strains of pneumococci after return to the usual type none developed endocarditis. In five the injection was combined with injury to the valves; four died of pneumococemia after the injection of large doses. Three animals were given a second injection and it was found that the first dose had produced a hypersensitiveness just as in animals injected with staphylococcus 334 (see page 251). Only one animal developed lesions by the injection of a relatively small dose. The injection of huge doses was necessary to produce pneumococemia when the organisms injected have grown in media free from blood or serum; the presence of the latter in broth in large amounts seemed to enhance virulence. Cultures from the animals that died with endocarditis always showed the above-mentioned special characteristics, while those that were obtained from animals dying of pneumococemia usually showed the organisms to have taken on the normal characteristics. A study of the blood and peritoneal exudate in some of the animals showed that the organisms disappeared rapidly which is in keeping with their susceptibility to phagocytosis. Blood cultures in four of the animals which developed active endocarditis were sterile 1, 2, 3, 4, and 5 days respectively before death or autopsy. The rapid destruction of the organisms even in the cases which later developed endocarditis was associated with apparent complete recovery for a time. The number of organisms in the blood after death was very large. When death occurred as the result of endocarditis or pericarditis it seemed to be due largely to mechanical obstruction to the flow of blood owing to large vegetations and to mechanical interference of the heart's action by the massive pericarditis.

Joint involvement occurred only once in all the animals inoculated.

Three of the strains that produced endocarditis were used to determine whether repeated inoculation would increase their virulence or their power of producing endocarditis. The virulence promptly became greater, death being caused by pneumococemia, the cocci taking on the usual or normal characteristics. Restored virulence of the cocci was associated with restored resistance to phagocytosis just as I found to be the case with pneumococci isolated from pneumonia.<sup>1</sup>

<sup>1</sup> *Jour. Infect. Dis.*, 1906, 3, p. 683.

## GENERAL SUMMARY.

A comparison of the number of times the sides of the heart were involved in the human cases and in the animals is interesting.

	Human	Animal
Left Side (alone)		
Mitral . . . . .	7 times	4 times
Mitral and aortic . . . . .	2 "	2 "
Aortic . . . . .	1 "	1 "
	10 "	7 "
Right Side (alone)		
Tricuspid . . . . .	2 "	4 "
Pulmonary . . . . .	1 "	1 "
	3 "	5 "
Right and Left Side		
Aortic and tricuspid . . . . .	1 "	2 "

It is to be remembered that the animals developed endocarditis without previous lesion of the valves. We see that the left side of the heart is more often involved in both series and proportionately more often in the human than in the animal cases. The mitral and tricuspid valves are more often involved than the aortic and pulmonary valves in both series; the right side proportionately oftener in the animals.

The greater frequency of involvement of the mitral and tricuspid valves, which contain capillaries, may be owing to embolic origin. This origin certainly occurred in one instance in the animals. That the right side should be involved proportionately more often than the left in the animals is in accord with expectations.

In nearly all cases of spontaneous endocarditis the bacteria which gain entrance into the circulation first must pass over the valves on the right side, consequently one would expect this side to be involved more frequently. Virchow suggested that the greater strain on the right heart in the fetus and on the left heart after birth is the cause of the predominance of endocarditis on these sides respectively during the two periods indicated. Now it has been shown that the property of adhering to the surface of agar and of forming clumps in broth of both the pneumococci and the staphylococcus was dependent to a large extent upon the quantity of oxygen present. Hence it would seem that the presence of arterial or oxygenated blood upon the two sides at the different periods may also serve to account for the peculiarities of localization, perhaps not by favoring the growth of the bacteria in question as Rosenbach believed, but by favoring the development of

the special characteristics which make the bacteria better able to attach themselves to the valve because of increased viscosity as well as to lodge in the minute capillaries of the valves because of the tendency to grow in clumps in the presence of abundant oxygen. This explanation is the more plausible because of the importance of these special characteristics in the production of endocarditis in animals.

The results of the observations recorded indicate that the bacteria in question, which seem to be of relatively little virulence in the ordinary sense, are able to maintain their growth in the blood and upon the endocardium, and ultimately cause death, by a process of immunization against the antibodies of the host. That the bacteria were not highly virulent for human beings in the ordinary sense seems certain because the course in all cases was a chronic one, which we would not expect were the organisms highly virulent. Furthermore, the bacteria upon isolation were all susceptible to phagocytosis in human serum by human leucocytes and were without pronounced pathogenic power for animals. Repeated injection made the animal more susceptible to subsequent injections instead of more resistant. The fact that huge doses were necessary to produce endocarditis in animals would seem to have its explanation in the fact that many of the bacteria were needed to make the tissues susceptible or give the bacteria a chance to adapt themselves to the new conditions.

The bacteria grew better and acquired a greater resistance to phagocytosis in the patient's serum than in the normal human serum and normal leucocytic blood had a much greater bactericidal power for the bacteria than the homologous patient's blood of the same leucocyte content. The bacteria consequently seemed to protect themselves by a process of adaptation to the opsonins and other antibodies of the individual host. Thus the opsonic index with normal leucocytes might be high while at the same time it might be low with the leucocytes of the patient. Again normal leucocytic blood might show no greater phagocytosis under comparable conditions than the patient's blood and the former might cause marked destruction of the bacteria and the latter none. Furthermore, normal leucocytes in the patient's serum and patient's leucocytes in normal serum might be actively bactericidal yet the patient's leucocytes and serum would cause no destruction. This acquired resistance to phagocytosis may be closely



dependent on some thermosensitive property of the serum because the staphylococcus and one strain of pneumococcus when grown in the heated serum of the patient remained susceptible to phagocytosis.

The animal experiments showed that the pneumococci if injected in large doses might cause death by pneumococcemia, especially if the animal had been given injections previously. At other times endocarditis might result. This was usually progressive but healing might also result. It must be emphasized that without the so-called special characteristics I was unable to produce endocarditis without previous injury to the valves. This holds good also for the staphylococcus. Then, again, injections in animals may produce no apparent effects.

There are, then, certain special conditions when endocarditis is readily produced without trauma of the valves.

#### CONCLUSIONS.

That certain bacteria more frequently attack the valves upon the left side of the heart than the right after birth, and the right more often than the left in the fetus, may be owing in part at least to the fact that abundant supply of oxygen favors the development of certain special characteristics of the bacteria that favor the production of endocarditis. Embolic origin may explain the greater frequency of endocarditis of the mitral and tricuspid valves as compared with aortic and pulmonary endocarditis.

A close relation exists between the biological characters of the bacteria and their ability to produce endocarditis in the class of cases observed.

The bacteria isolated, while having little or no pathogenic power to animals and being susceptible to phagocytosis, present definite evidence of being immunized against the antibodies of the individual host, thereby perhaps overcoming the resistance of the latter.

The injection of dead bacteria in endocarditis has little or no influence upon the course of the disease. Late in the course temporary improvement may follow the injections.

Blood cultures are the best means of making an early diagnosis in acute endocarditis. They should always be made for the identification and study of the infecting organism as well as for prognostic reasons.



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## THE SUSCEPTIBILITY OF GOPHERS, FIELD MICE, AND GROUND SQUIRRELS TO PLAGUE INFECTION.\*<sup>1</sup>

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THIS investigation was begun with the hope of determining the susceptibility to *B. pestis* of all of the small mammals found in the vicinity of San Francisco, but this was found to be impracticable on account of the difficulty in procuring the necessary animals and in keeping them. Many of the animals obtained died in captivity. It was found, however, that gophers (*Thomomys bottae*), field mice (*Microtus californicus*), and ground squirrels (*Citellus beechyi*) ordinarily stood confinement very well.<sup>2</sup> We have kept a number of each of these species for several months and they were found to remain in perfect health. Any deaths among these animals will probably occur in the first week of captivity; therefore, to avoid confusion in our work we have used for our experiments only those animals that had been kept in stock for a week or longer. Hares (*Lepus*——), moles (*Scapanus californicus*), shrews (*Sorex*——), and weasels (*Putorius xanthogenys*) were frequently captured but they never

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<sup>1</sup> This work was undertaken at the Plague Laboratory of the Public Health and Marine Hospital Service at San Francisco, Cal., at the suggestion of Surgeon M. J. Rosenau, Director of the Hygienic Laboratory, Washington, D. C.

<sup>2</sup> Stevens, *California Mammals*, San Diego, 1906.

lived long enough to make it practicable to use them for experimental purposes.

CALIFORNIA POCKET GOPHERS (*Thomomys bottae*).

These animals were inoculated by the cutaneous method with tissues from naturally or artificially infected plague rats, as is shown in the following table.

TABLE 1.

Gopher	Materials Used for Inoculation	Day of Death	Day Killed	Lesions	Remarks
No. 1 . . . . .	Spleen natural plague rat No. 64	..	7	None	
No. 2 (small)	Spleen natural plague rat No. 66	3	..	Slight gen. injection	Smears suspicious
No. 3 (small)	Spleen natural plague rat No. 66	..	6	None	
No. 4 (large)	Spleen natural plague rat No. 66	..	10	None	
No. 5 (small)	Spleen of inoculated rat No. 8	4	..	Plague	Liver of this gopher used to inoculate a guinea-pig. The guinea-pig died on the 5th day with typical lesions of plague and a pure culture of <i>B. pestis</i> was isolated from the liver of the latter
No. 6 (large)	Spleen of inoculated rat No. 8	..	8	None	
No. 7 (small)	Spleen of inoculated rat No. 8	..	8	None	

The lesions in the case of No. 5 were an extensive sero-gelatinous exudate at the site of the inoculation and over the whole front of the body; the left inguinal gland was large, hard, and reddish in color, but not caseous. Pest-like organisms were found in the smears made from the sero-gelatinous exudate, the enlarged gland, and from the liver and the spleen. The fact that tissue from this animal conveyed infection to a guinea-pig proved conclusively that the gopher was infected.

It should be mentioned that virulent cultures of *B. pestis* were isolated from the animals that furnished the material for these inoculations: i. e., Rat 64,<sup>1</sup> Rat 66, and inoculated Rat 8.

Of seven gophers inoculated by the cutaneous method, one (No. 5) certainly died of plague; one (No. 2) probably died of plague. It is unfortunate that a guinea-pig was not inoculated from this animal (No. 2), but on account of the limited space available for keeping inoculated animals this was not practicable.

In another experiment, three gophers were inoculated by the subcutaneous method with 0.5 c.c. of an emulsion of the spleen of

<sup>1</sup> Throughout this paper "rat" refers to *Mus norvegicus*.

a guinea-pig dead of acute plague due to inoculation with the spleen from a case of human plague. One of these gophers died within 24 hours after inoculation, showing at the post-mortem examination a reaction at the site of injection; the other two died about 48 hours after inoculation, and presented at necropsy an enlargement of the spleen and an extensive sero-gelatinous edema over the front of the body. From the liver of one of the latter animals a pure culture of *B. pestis* was obtained.

The following was the most convincing experiment in demonstrating the relative immunity of gophers to infection with *B. pestis*. Each of the animals mentioned in the following table was inoculated subcutaneously with one cubic centimeter of suspension in physiological salt-solution of a three-day agar culture of a virulent strain of *B. pestis*. However, the dose of culture suspended in the medium inoculated into the gopher was 100 times as great as that given the rat or the guinea-pig.

TABLE 2.

Animal	Weight of Animal in Gm.	Dose of Culture	Day of Death	Lesions
Guinea-pig ..	285	.0001 loop	4	Acute plague
Rat .....	280	.0001 "	4	Acute plague
Gopher .....	135	.01 "	Killed 9th day	None

A culture from the liver of the gopher remained sterile, and a guinea-pig inoculated from the spleen remained healthy.

#### FIELD MICE (*Microtus californicus*).

These animals appear to be fairly common in the vicinity of San Francisco, as considerable numbers are brought to the laboratory along with the rats from the outlying parts of the city. The average weight of an adult field mouse is about 40 gm.

**Experiment 1.**—One loopful of a five-day-old virulent agar culture of *B. pestis*, sixth generation, from a plague rat, was used to inoculate each of five field mice by the cutaneous method. The results are shown in the following table:

TABLE 3.

Field Mouse	Day of Death	<i>B. pestis</i> Isolated from Liver
No. 1.....	4	Yes
No. 2.....	4	Yes
No. 3.....	6	No
No. 4.....	Well on 24th day	
No. 5.....	Well on 24th day	



The lesions presented by Nos. 1, 2, and 3 were those mentioned under Exp. 2 (below). Animals No. 4 and No. 5 were alive and well on the 24th day, at which time they were given subcutaneously the same amount of the same culture that proved fatal to the five field mice and the guinea-pig in Experiment 2. Five days later these two animals were chloroformed while they were apparently in perfect health. No lesions were found and smears were negative. I am disposed to believe that these animals were infected by the first (cutaneous) inoculation but recovered and thereby developed an immunity that saved them from the second (subcutaneous) inoculation.

**Experiment 2.**—Five of the animals were inoculated subcutaneously with a highly virulent culture of *B. pestis* (sixth generation on artificial media) derived from a natural plague rat. Each one was given 1/100 of a loopful of a 24-hour agar culture suspended in physiological salt-solution. One died on the second day (48 hours), three on the third day, and one on the fourth day. The lesions somewhat resembled the lesions of plague in rats. There was a more or less marked general subcutaneous injection and some thickening at the site of inoculation. In only one case was a well-defined bubo present. The spleen was always enlarged and firm. The liver was usually yellowish white instead of the normal brown. A pure culture of *B. pestis* was recovered from the liver of each animal. As a control a guinea-pig weighing 567 gm. was inoculated at the same time with the same dose of the culture. This guinea-pig died on the seventh day with typical lesions of plague.

**Experiment 3.**—Three field mice were inoculated by the cutaneous method with one loopful of a 24-hour agar culture, first generation, of *B. pestis* isolated from one of the field mice that died on the third day, in the preceding experiment. One died on the third day but as it had been partly mutilated by its companions in the cage, the post-mortem examination was unsatisfactory; however, a general subcutaneous injection was found and I think it may be assumed that this animal died of plague. The other two animals were killed on the 10th day. They presented no lesions. Smears and cultures were negative.

**Experiment 4.**—The last experiment with field mice was undertaken to determine whether plague infection could be successfully carried through a series of these animals.

*1st Passage.*—Three field mice were inoculated by the cutaneous method with the spleen from a case of natural rat plague. One died on the third day, presenting the lesions previously noted as due to plague in these animals, and in addition, on the surface of the intestines there were small subserous hemorrhages. The two remaining animals were killed on the eighth day. One of them presented no lesions; the other had an enlarged spleen, and a caseous bubo in one groin in the contents of which were many pest-like organisms (chronic plague).

*2d Passage.*—The spleen of the first of the preceding animals (dead on third day) was used to inoculate two field mice by the cutaneous method. One of these died on the third day. Cultures from the liver remained sterile but there were the gross lesions usually found in plague in these animals, and in addition whitish granules in the liver. The other died on the fourth day and presented similar lesions at autopsy.

*3d Passage.*—The spleen of the first of the mice in the second passage was used to inoculate (cutaneous method) another field mouse. This animal died on the third day presenting the usual lesions of plague at necropsy, and had in addition a small, clear, serous, pleural effusion. The experiment had to be discontinued at this point owing to the lack of animals for continuing the series.

GROUND SQUIRRELS (*Citellus beechyi*).

Since this work was undertaken the common ground squirrel of California (*Citellus beechyi*) has been found infected with plague in nature,<sup>1</sup> and several cases of plague in man have been clearly traced to infection in these rodents.

Pioneer work on the experimental infection of these animals was done in 1904 by Passed Assistant Surgeon D. J. Currie, U.S.P.H. and M.H.S., who found them very susceptible to plague infection. Dr. Currie succeeded in infecting these rodents by feeding, by cutaneous and subcutaneous inoculation, and by "contact."

In the course of work now in progress with these animals we have had occasion to inoculate 19 of them by the cutaneous method, and in each case the animal has died of plague within six days of the time of inoculation. The lesions are a large slough at the site of inoculation, a caseous bubo, and an enormously enlarged spleen.

In regard to the cutaneous method of inoculation used in the experiments reported here it is proper to state that the technique was to shave the skin of the abdomen so that a raw surface was presented into which culture or tissue was rubbed. As is well known this method is a very reliable one for infecting animals with *B. pestis*. It practically never fails with guinea-pigs and white rats.

## SUMMARY AND CONCLUSIONS.

Gophers are highly resistant to plague when inoculated by the cutaneous method, but apparently often susceptible when inoculated subcutaneously. As only four animals were used for the subcutaneous inoculation, no sure conclusion can be drawn from the experiments.

Field mice are moderately susceptible to cutaneous inoculation and quite susceptible to subcutaneous inoculation. Plague infection was successfully carried directly from animal to animal through three transfers in field mice by the cutaneous method of inoculation.

Ground squirrels are highly susceptible to plague infection, no example of immunity having been encountered.

We may conclude that gophers are not sufficiently susceptible to infection with *B. pestis* to be of any importance from an epidemiological point of view. Field mice are probably about as susceptible

<sup>1</sup> Wherry, *Jour. Infect. Dis.*, 1908, 5, p. 485.

as rats, but as they rarely come in close contact with man, it is improbable that they will ever be a serious factor in the spread of plague. As we already know from experience, ground squirrels are of the utmost importance in plague epidemiology.

The animals used in these experiments represent three families of the order Rodentia, as follows:<sup>1</sup>

*Citellus beechyi* . . . . . Family Sciuridae

*Microtus californicus* . . . . . Family Muridae

*Thomomys bottae* . . . . . Family Geomyidae

<sup>1</sup> Elliott, "A Synopsis of the Mammals of North America and the Adjacent Seas," Field Columbian Museum, Chicago, 1901

## THE IMMUNITY OF SAN FRANCISCO RATS TO INFECTION WITH *B. PESTIS*.\*

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EXPERIMENTS undertaken for the purpose of ascertaining the influence upon the virulence of *B. pestis* by passage from rat to rat without intermediate culture on artificial media showed that a certain number of the wild rats (*Mus norvegicus*) in San Francisco were immune to plague infection. This result led me to undertake a series of experiments to determine the extent of this immunity.

The Indian Plague Commission<sup>1</sup> found that 59 per cent of the wild rats of Bombay were immune when inoculated by the **cutaneous** method with splenic material containing a large number of *B. pestis*. They also found a certain percentage of the rats immune to **subcutaneous** inoculation of agar culture of *B. pestis*. The smallest dose of plague culture used by the Indian Commission in their work was  $\frac{1}{250}$  part of an agar culture. This immunity varied from 3.7 per cent to 10.5 per cent, depending upon the amount of culture injected. They note that other writers had previously observed the immunity of wild rats to plague infection.

It is somewhat difficult to determine just what constitutes complete immunity in the case of animals in which careful clinical observation is impracticable, as it is very evident that an animal may have a slight infection of which no trace remains when it is examined a week or 10 days after the inoculation. It was assumed in my work that a rat that had not died by the eighth day would not die of acute plague, and that the lesions of chronic plague would be sufficiently developed to offer no difficulty of diagnosis.

The diagnosis of acute plague was based upon the gross lesions observed at post mortem, and in a few cases where the gross lesions were not sufficiently characteristic, cultures were made, and *B. pestis* isolated. The lesions of chronic plague, a caseous or purulent lym-

\* Received for publication April 17, 1909.

<sup>1</sup> *Jour. of Hyg.*, 1906, 6, p. 422.

phatic gland alone or associated with large caseous foci in the spleen, or such foci in the spleen without other lesions, offer more difficulty in diagnosis. Some of the cases diagnosed as chronic plague have been verified by inoculation into a guinea-pig of the material from the bubo or the spleen. In the other cases, the diagnosis has been based upon the gross lesions alone. I may remark here that in the examination of considerably over 100,000 rats in San Francisco, no case that could be proven to be chronic plague due to natural infection has been encountered, and out of all those examined not more than half a dozen rats have been seen presenting lesions strongly suggestive of chronic plague as we have observed it in inoculated rats.

It is, of course, very evident that results in an investigation of this sort will depend very largely on the virulence of the organism used. For this work, when cultures were employed we have used only those known to be highly virulent. The tissues used are known to have been capable of producing acute plague which was invariably fatal in all of the guinea-pigs and all of the white rats that were inoculated.

I am unable to say exactly what influence the size of the dose (aside from overwhelmingly large doses) of culture inoculated has in determining whether infection in the rat will take place or not. It is evident that the existence of natural immunity among animals makes it very difficult to determine this point. There is practically no natural immunity to plague infection among guinea-pigs and in these animals the size of the dose of culture used is a matter of but little consequence. Indeed it happens not infrequently that a guinea-pig having received a certain amount of culture of *B. pestis* will die earlier than one having received a very much larger dose of the same culture.

The only experimental evidence bearing on this point in reference to the wild rats of San Francisco is as follows: A culture from natural plague Rat 64, third generation on agar, was used to inoculate subcutaneously four medium-sized rats (*Mus norvegicus*).

It will be seen here that Rat 2, which survived, received a dose of culture 100 times as great as that given Rat 4, which died on the fifth day with lesions of acute plague. *B. pestis* was isolated from



the liver of Rat 4. Rat 2 was killed on the ninth day and was found to be entirely normal except for an enlarged spleen. Cultures from the spleen remained sterile. I am disposed to believe that this animal had suffered from a mild infection from which it was recovering, the enlarged spleen being the only remaining evidence of the contest between the bacteria and the defensive agencies of the body.

TABLE 1.

	Weight of Rats Gm.	Dose of Culture Used			Result
Rat 1 .....	175	0.01	loop	agar culture	Died 4th day of acute plague
" 2 .....	155	0.0001	"	" "	Killed 9th day; enlarged spleen only
" 3 .....	185	0.00001	"	" "	Died 8th day of subacute plague
" 4 .....	175	0.000001	"	" "	Died 5th day of acute plague

Very large doses of culture frequently kill the animals early without producing characteristic lesions of plague infection.

In the early part of my work it was observed that the older rats were much less susceptible to infection than the younger ones; therefore, whenever practicable a record was made of the weight of each animal at the time of inoculation, and as will be seen throughout the tables, the percentage of immunity is much higher in old (large) rats than in young (small) ones.

**Technic.**—Our inoculated rats were kept in specially constructed insect-proof cages, and under such conditions that there was practically no natural mortality among them. Occasionally a rat died in the cage from some cause other than plague, but this was very exceptional. When the subcutaneous method of inoculation was used, the suspension of the culture in physiological salt-solution was injected in the usual manner under the skin of the abdomen. When the cutaneous method was employed, the skin of the belly was shaven dry, and care was always taken to abrade the epithelium, leaving a raw surface. The culture was rubbed into the raw surface with a platinum loop, or if tissue was used it was vigorously rubbed on the abraded area.

The record of the experiments will be arranged here under the head of the species of rats used.

*Mus alexandrinus.*

We have been able to secure but one of this species alive. This specimen weighed 70 gm. and was inoculated subcutaneously with 1/100 of a loop of an agar culture of *B. pestis* which had been isolated about eight months previously. The animal died on the fourth day and presented the typical lesions of plague. A culture of *B. pestis* was isolated from the liver. A guinea-pig control also died on the fourth day with typical lesions of plague.

*Mus rattus.*

Comparatively few rats of this species have been available for this work. The following table shows the results of the experiments in tabular form.

TABLE 2.

MATERIAL USED FOR INOCULATION	DOSE AND MODE OF INOCULATION	NO. RATS USED	WEIGHT GM.	DIED OF ACUTE PLAGUE DAY OF DEATH			
				3	4	5	6
Agar culture from squirrel. . . . .	0.01 loop subcut.	3	All small	2	1	..	..
Agar culture from <i>M. norvegicus</i> . . . . .	0.01 " "	2	90-?	..	1	..	1
Agar culture from <i>M. rattus</i> . . . . .	0.01 " "	2	105-250	..	1	1	..
Agar culture from <i>M. norvegicus</i> . . . . .	1 " cutan.	1	Large	Killed on 9th day; chronic plague			

It will be observed that of the eight rats used all died of acute plague excepting one which was killed on the ninth day after inoculation and found to have chronic plague. A guinea-pig inoculated from the spleen of this rat died of acute plague.

The cultures used had all been isolated directly from naturally infected animals. The squirrel and the *Mus rattus* cultures were only a few days old. The *Mus norvegicus* cultures were about five months old. The number of rats used was not large enough to enable one to draw any conclusions, but no example of absolute immunity was encountered. The case of chronic plague may perhaps be regarded as an example of partial immunity.

*Mus norvegicus.*

The first experiments were with material derived directly from cases of natural plague. The spleen from a case of human plague was used to inoculate two rats by the cutaneous method. One died on the seventh day and was found to present lesions of acute plague; the other (the larger) was killed on the seventh day and found entirely normal.

Three medium-sized rats were inoculated by the cutaneous method with the spleen of a natural plague rat. One died on the fifth day, one on the sixth; both showed the lesions of acute plague; the third, when killed on the ninth day, was found to present the lesions of chronic plague.

Three grown rats were inoculated subcutaneously with an emulsion of the spleen from a plague rat (a pure culture of *B. pestis* was isolated later from the spleen). One died of typical plague on the third day. The other two were killed on the sixth day. One showed a local abscess at the site of inoculation; cultures from the spleen remained sterile. The third showed a caseous bubo only in one groin. The bubo was used to inoculate a guinea-pig by the cutaneous method. The guinea-pig died of acute plague on the sixth day.

Control guinea-pigs and white rats were inoculated in each case with the same material used to inoculate the wild rats. These controls all died of acute plague.

The next experiment extended over a period of about four months. The culture used was one isolated on June 30, 1908, directly from the liver of a natural plague rat. It had never been passed through a laboratory animal. This culture is very virulent or guinea-pigs and white rats. The fifth generation on agar was kept as a stock culture and from this a subculture was made when we wished to inoculate a series of rats so that each series was inoculated with the sixth generation of this culture. The platinum loop used was one that was estimated to be capable of taking up 260,000,000 *B. pestis* from an agar culture. One ten-millionth part of this loopful of culture injected sub-

cutaneously killed in eight days a guinea-pig weighing over 300 gm. The dose used for the rats in these experiments was 100,000 times as great.

A 24-hour culture was used except in the series inoculated on October 12, when a five-day-old culture was used.

TABLE 3.

DATE OF INOCULATION	DOSE AND MODE OF INOCULATION	NO. RATS USED	MAX. AND MIN. WEIGHTS GM	DIED OF ACUTE PLAGUE DAY							KILLED 10TH DAY OR LATER	
				2	3	4	5	6	7	TOTAL	Chronic Plague	Normal
1908												
Aug. 27.....	0.01 loop subcut.	6	74-110	0	5	1	0	0	0	6	..	..
" 27.....	0.01 " "	6	280-442	0	1	3	1	0	0	5	..	1
Sept. 9.....	0.01 " "	6	37-85	0	1	5	0	0	0	6	..	..
" 9.....	0.01 " "	6	205-345	0	3	1	0	0	0	4	..	2
" 10.....	0.01 " "	6	50-85	0	0	3	3	0	0	6	..	..
" 10.....	0.01 " "	6	190-245	0	0	4	1	0	1	6	..	..
" 23.....	0.01 " "	4	70-105	1	1	2	0	0	0	4	..	..
" 23.....	1 " cutan.	4	50-105	0	2	1	1	0	0	4	..	..
" 23.....	0.01 " subcut.	4	225-350	0	3	0	1	0	0	4	..	..
" 23.....	1 " cutan.	4	160-340	0	0	0	1	0	0	1	..	3
Oct. 1.....	0.01 " subcut.	4	80-95	1	2	1	0	0	0	4	..	..
" 1.....	1 " cutan.	4	80-115	0	0	4	0	0	0	4	..	..
" 1.....	0.01 " subcut.	2	200-290	0	0	1	1	0	0	2	..	..
" 1.....	1 " cutan.	6	170-270	0	0	1	2	0	0	3	..	3
" 12*.....	1 " "	6	80-115	0	4	1	0	0	1	6	..	..
" 12*.....	1 " "	3	165-190	0	0	0	2	0	0	2	1	..
Dec. 28.....	0.01 " subcut.	2	215-250	0	1	0	0	0	0	1	..	1
" 28.....	1 " cutan.	2	190-210	0	0	0	0	0	0	0	..	2
" 28.....	0.01 " subcut.	8	55-130	0	1	2	4	1	0	8	..	..
Total.....		89		2	24	30	17	1	2	76	1	12
Summary {	0.01 loop subcut.	34	37-130	..	..	..	..	..	..	34	..	..
	1 " cutan.	14	50-115	..	..	..	..	..	..	14	..	..
	0.01 " subcut.	26	190-442	..	..	..	..	..	..	22	..	4
	1 " cutan.	15	160-340	..	..	..	..	..	..	6	1	8
Grand total		89		2	24	30	17	1	2	76	1	12

\* Five-day-old culture.

A number of rats have been inoculated by the cutaneous method from the tissues of squirrels infected experimentally with plague. The material used for inoculation always showed large numbers of pest-like organisms. Guinea-pig controls were inoculated at the same time and in the same manner as the rats, one guinea-pig being used for every three rats. The controls all died of acute plague. The results are shown in the following table:

TABLE 4.

	NO. USED	DIED OF ACUTE PLAGUE DAY OF DEATH				DIED OF CHRONIC PLAGUE OR FOUND SUFFERING FROM SAME WHEN KILLED	KILLED 10TH DAY OR LATER; NO LESIONS
		4	5	6	7		
Rats weighing under 125 gm. ....	27	5	8	3	3	2	6
Rats weighing over 200 gm. ....	3	..	..	1	..	1	1

Sixteen small rats and eight large ones were inoculated by the cutaneous method with the spleen of a guinea-pig dead of typical acute plague on the seventh day after inoculation with material from an artificially infected squirrel. The smears from the

spleen showed large numbers of typical *B. pestis* and a pure culture of the organism was isolated from it. The results were as follows:

TABLE 5.

	No. USED	DIED OF ACUTE PLAGUE DAY OF DEATH				CHRONIC PLAGUE; DIED AFTER 9TH DAY OR LESIONS FOUND WHEN KILLED ON 14TH DAY	KILLED ON 14TH DAY; NO LESIONS
		4	5	6	7		
Rats weighing under 120 gm.....	16	1	2	1	2	4	6
Rats weighing over 295 gm.....	8	0	0	0	0	2	6

A number of rats have been inoculated by the cutaneous method from rats artificially infected with plague. Unfortunately no accurate record was made of the weight of the animals inoculated; they are simply classified as large, small, and medium. The results are as follows:

TABLE 6.

	NUMBER INOCU- LATED	DIED OF ACUTE PLAGUE DAY OF DEATH						CHRONIC PLAGUE	NO LESIONS
		2	3	4	5	6	7		
Large rats .....	12	..	1	2	3	..	..	4	2
Medium-sized rats .....	9	1	3	1	2	..	1	..	1
Small rats.....	5	..	..	1	1	2	..	..	1

Combining the figures in the last three tables, which may properly be done as the conditions were similar, we find that the results are as follows:

TABLE 7.

	No. Used	Acute Plague	Chronic Plague	No Lesions
LARGE RATS				
Table 4.....	3	1	1	1
Table 5.....	8	0	2	6
Table 6.....	12	6	4	2
Total.....	23	7 (30+ per cent)	7 (30+ per cent)	9 (39+ per cent)
SMALL RATS				
Table 4.....	27	10	2	6
Table 5.....	16	6	4	6
Table 6.....	5	4	0	1
Total.....	48	20 (60+ per cent)	6 (12.5 per cent)	13 (27+ per cent)

The immunity demonstrated in these experiments I regard as natural and not as acquired, for the following reasons:

First, the percentage of immunity in old rats is so high as to preclude the possibility of a previous attack of plague in these cases. The natural plague infection among San Francisco rats has never exceeded 2 per cent and this high percentage existed for a very short time only.

Second, a considerable percentage of immunity to inoculation by the cutaneous method was found in rats that were certainly not more than two or three months old. These rats were used six months after the epizootic had terminated. In other words, an immunity was found among rats that we know positively had never been exposed to an epizootic of plague.

#### SUMMARY.

A considerable immunity to plague infection exists among the wild rats of San Francisco. The percentage of immunity is especially high among the old rats.

The immunity encountered is probably natural in most cases, not acquired.

NOTE.—The three species of rats may readily be distinguished by attention to the following points:

*Mus norvegicus* (Norway rat, sewer rat, gray rat; until recently known as *Mus decumanus*).—This constitutes about 98 per cent of the total rat population of San Francisco. The color is gray or brownish. The under surface of the body is usually gray or grayish brown, but occasionally it will be almost pure white. At times specimens are encountered in which large, pure white spots are found on the abdomen. The head is short and broad. The ears are rather short. The tail is shorter than the head and the body together.

*Mus rattus* (black rat, house rat, ship rat).—This species is usually smaller than *Mus norvegicus*. The color is black, sometimes with a grayish tinge. The belly is usually black, but sometimes white. The head is more pointed than in *Mus norvegicus*. The ears are much larger and more delicate than those of *Mus norvegicus*. The tail is longer than the head and the body together.

*Mus alexandrinus*.—This rat is regarded by some writers as merely a variety of *Mus rattus*. It is often spoken of as the roof rat or tree rat. It resembles *Mus rattus* rather closely except in color, which is grayish red or rusty. The belly is pure white. The tail is always longer than the head and body.

A good many specimens are encountered in which there is difficulty in distinguishing *Mus alexandrinus* from *Mus rattus*, but *Mus norvegicus* is not likely to be confused with either of the other species. We have seen no specimen that we had any reason for considering a hybrid.



# IMMUNOLOGICAL OBSERVATIONS IN ULCERATIVE CYSTITIS CAUSED BY PSEUDODIPHTHERIA BACILLUS.\*

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**History of the case.**—Woman, typesetter, unmarried, 23 years of age, no history of tuberculosis or hereditary diseases in family; never robust, always of good health except for nervousness and severe pain during menstrual periods. About two years ago began to have a slight vaginal discharge; for this and for the painful menstruation, the uterus was curetted. Did not improve and grew very nervous. Six months later both tubes and ovaries were removed; immediately thereafter intense vaginal pain and a profuse vaginal discharge developed. Gradually she improved and returned to work, but has never been entirely free from distress and discharge since. Six months ago (December, 1907) she was taken with a severe chill followed by painful, frequent, and bloody micturition, tenesmus, fever, and prostration. Since then, better and worse at times. Has lost weight, but worked constantly. No cough or expectoration at any time. A cystoscopic examination by Dr. J. Clarence Webster, to whom I am indebted for the opportunity to study the case, showed a large irregularly ulcerated area, approximately  $4 \times 5$  cm., upon the posterior wall of the bladder behind the trigone; the base of some of the ulcers were irregular, grayish, and necrotic patches, while others were clean. The ureteral orifices free. The vagina atresic and in the vault was a pocket containing thick pus. This was opened and drained, vagina packed with chynesol gauze. The patient left the hospital temporarily because it was thought unwise to drain the bladder before the vaginal condition improved.

June 8, 1908: Two days after cystoscopic examination; the patient is suffering excruciating pain in the bladder and back associated with a constant desire to urinate, extreme tenesmus, and burning. No distinct evidence of involvement of kidneys or of renal colic can be made out although she complains of severe pain in the back. The pulse is normal; no fever. The 24-hour specimen of urine, 720 c.c., is turbid but not bloody, alkaline, specific gravity 1.015, containing a large amount of albumin but no casts, a few leukocytes and epithelial cells. Stained specimens of sediment show gram-negative, moderately motile bacilli and a fair number of gram-positive bacilli resembling the diphtheria group. The fresh single specimen also shows a large amount of albumin, no casts, some leukocytes showing various stages of disintegration, and a large amount of albuminous debris. Stained specimens of the sediment show about an equal number of the bacilli mentioned.

June 10, 1908: A freshly catheterized specimen was plated out on plain and urine agar. Two varieties of bacilli developed: One gram-negative moderately motile that proved to be the colon bacillus on further cultivation. The other was a gram-positive bacillus showing a rather marked tendency to polymorphism and irregular staining. The same bacilli were again found in the sediment.

\* Received for publication March 10, 1909.

June 15, 1908: Catheterized specimen of urine was thoroughly centrifugated and the sediment was found to contain a fair number of leukocytes, few epithelial cells, no gram-negative bacilli but a rather large number of gram-positive bacilli. The cultures on Löffler's blood serum gave a luxuriant growth at the end of 24 hours of the diphtheria-like bacillus. These bacilli were always more numerous in a 24-hour specimen or in a single specimen which had stood for a period. On the other hand the number of leukocytes and epithelial cells was much greater in a fresh specimen than in one which had stood for a time. In order to explain the apparent solution of these cellular elements, some of the urine was made sterile by filtration and used as a culture medium. The pseudodiphtheria bacillus grew rather luxuriantly in it. The urine constantly contained a rather large amount of albumin which presumably furnished the necessary nourishment. A suspension of washed leukocytes and epithelial cells (from another case of cystitis) were now mixed in equal quantity with fresh filtered urine and urine filtered after bacilli had been grown in it for 24 hours, placed in the incubator after determining the number of cells by means of a leukocyte counter and left for 12 and 24 hours. A careful estimate of the number of cells intact at the end of these periods, showed that there was practically no solution of the cells in the urine filtered when fresh, while there was a marked diminution in the urine in which the bacilli had grown for 24 hours.

TABLE 1.  
THE EFFECT OF THE URINE UPON LEUKOCYTES AND EPITHELIAL CELLS.

	No. of Leukocytes and Epithelial Cells per c mm		
	Immediately	12 Hours	24 Hours
Fresh filtered urine.....	250	225	200
Urine filtered after bacillus had grown in it for 24 hours.....	325	115	4

Apparently the bacillus growing in the urine produced substances that caused the solution of the cells. A control experiment with normal urine failed because the bacillus did not grow in sufficient amount.

The patient returned to the hospital for further study on June 16, 1908. The temperature during an exacerbation of the bladder symptoms went up to 100° F., otherwise it was normal except for a rise of one degree for several days following June 26, on which day a vesico-vaginal fistula was made. A profuse hemorrhage followed. The drainage of the bladder relieved the suffering, the general condition improved rapidly, and she left the hospital three weeks later. She received 50 million killed bacilli on June 14, 150 million on July 4, and 500 million on July 9, 1908.

Smears from the scrapings from the margin of the ulcerated area showed large numbers of gram-positive bacilli, epithelial cells, and leukocytes. There was no evidence of phagocytosis. Gram-positive bacilli were also present in smears made directly from the granulating surface from the vault of the vagina. Cultures from the scrapings as well as from the vagina yielded the diphtheria-like bacillus in predominating numbers, only a few colon bacilli and staphylococcus colonies developed. Repeated examination of the urine and of the scrapings directly from the ulcerated area in the bladder for tubercle bacilli gave negative results. A conjunctival tuberculin test gave no reaction. Occasional specimens of urine for eight months longer showed a gradual diminution in the number of leukocytes and albumin and a total disappearance of the gram-positive bacilli. The patient's general condition and local improvement were also marked.

The following experiments were made with the bacillus:

Guinea-pig 1, June 15, 1908: Surface growth of one Löffler's blood-serum slant intraperitoneally.

June 16: Very ill. Peritoneal smears rich in leukocytes and bacilli; moderate phagocytosis.

June 20: Lost much in weight. Peritoneal smears still contain gram-positive bacilli and leukocytes.

Gradual recovery. Chloroformed one month later. No lesions.

Guinea-pig 2: Blood-agar slant intraperitoneally. Death in six days. No peritonitis; blood cultures yielded few colonies of the bacillus.

Guinea-pig 3: Blood-agar slant intraperitoneally and 1,000 units of diphtheria antitoxin subcutaneously. Death in 24 hours. Peritoneal and blood smears rich in gram-positive bacilli. Pure culture isolated from both blood and peritoneum.

Guinea-pig 4: June 23, 1908: Surface growth of large plain-agar slant and of Löffler's blood-serum slant intraperitoneally. Grew ill June 29 and died. No bacilli could be found.

It is seen from the results of the experiments that the bacillus was one of moderate virulence and that diphtheria antitoxin failed to protect against the organism as has been the case with other virulent pseudodiphtheria bacilli. Inasmuch as the growth upon various media resembled closely that of diphtheria bacilli especially after they have been cultivated for a time, and for the reason that antitoxin failed to protect, it would seem justifiable to regard the bacillus as a so-called virulent pseudodiphtheria bacillus.<sup>1</sup>

**Phagocytic experiments.**—In a previous paper<sup>2</sup> I pointed out that leukocytes from pneumonia and other infections may be more active phagocytically than normal leukocytes. The phagocytic activity of leukocytes has been further studied in cases of endocarditis and colon bacillus pyelitis. In these conditions the leukocytes may be more or less active than normally. I have repeatedly observed that while a patient's opsonic index with normal leukocytes may be high, it may be below normal with the homologous leukocytes. In the present case a comparative study of the opsonic power of the patient's serum and the phagocytic activity of the leukocytes was made.

Table 2 illustrates the method by which the results given in the charts were obtained. The experiment was made June 22, 1908. The bloods which furnished the leukocytes were collected in 2 per cent sodium-citrate solution at practically the same time, and washed twice in at least 40 times the amount of isotonic salt solution, this

<sup>1</sup> Ruediger, *Trans. Chicago Path. Soc.*, 1903, 6, p. 45; Hamilton, *Jour. Infect. Dis.*, 1904, 1, p. 690; Hamilton and Horton, *ibid.*, 1906, 3, p. 128.

<sup>2</sup> *Jour. Infect. Dis.*, 1906, 3, p. 683.

being sufficient to remove the opsonins completely because when the washed leukocytes were suspended in salt solution and mixed with bacterial suspension no phagocytosis resulted. The bacterial suspension was made in salt solution from growths on Löffler's blood serum. The clumps were removed and the proper density obtained by centrifugation. The number of leukocytes indicated in the table was obtained by counting the leucocytes in the blood cream used in the experiment. Differential leukocyte counts were made often enough to determine that not only was the total number of leukocytes approximately the same but also that the polymorphonuclears were present in equal numbers in the blood creams used.

The amount of phagocytosis indicated in Table 2 was determined by counting the number of bacteria in at least 50 leukocytes and obtaining an average. The results given in Charts 1 and 2 were obtained on the dates indicated from experiments identical with the one given in Table 2.

TABLE 2.  
EFFECT OF NORMAL AND PATIENT'S LEUKOCYTES AND SERUM ON PSEUDODIPHtheria BACILLUS.

Washed Leukocytes, Serum, and Bacillary Suspension—Equal Parts	No. of Leukocytes per c.mm	Phagocytosis per Leukocyte After 15 Min.	Remarks
Normal leukocytes + normal serum.....	15,300	4.1	No agglutination
" " " + patient's serum.....	15,300	5.27	Moderate agglutination of bacilli
Patient's leukocytes + normal serum....	14,700	4.2	No agglutination
" " " + patient's serum....	14,700	2.05	Moderate agglutination of bacilli and leukocytes

The solid heavy line in Chart 1 represents the opsonic index as determined in the usual way, i. e., with normal leukocytes. It is above normal upon three successive days and practically normal on the first injection. The following day it rises to 1.4 to drop to 0.7 the second day after the injection; after the next two injections it remained almost normal.

The heavy broken line represents the opsonic index of the patient's serum with the patient's leukocytes. It is constantly below normal except on the first and second day after each injection, at which time it shows a sharp rise and each time going higher than the index obtained with normal leukocytes. Is this increase in phagocytosis following the injections the result of an increased activity of leukocytes or of opsonin, or of both combined? In order to decide these points,

Chart 2 was prepared. Table 2 shows that by dividing the result obtained with normal leukocytes and normal serum by the results obtained with the patient's leukocytes and normal serum (Lines 1

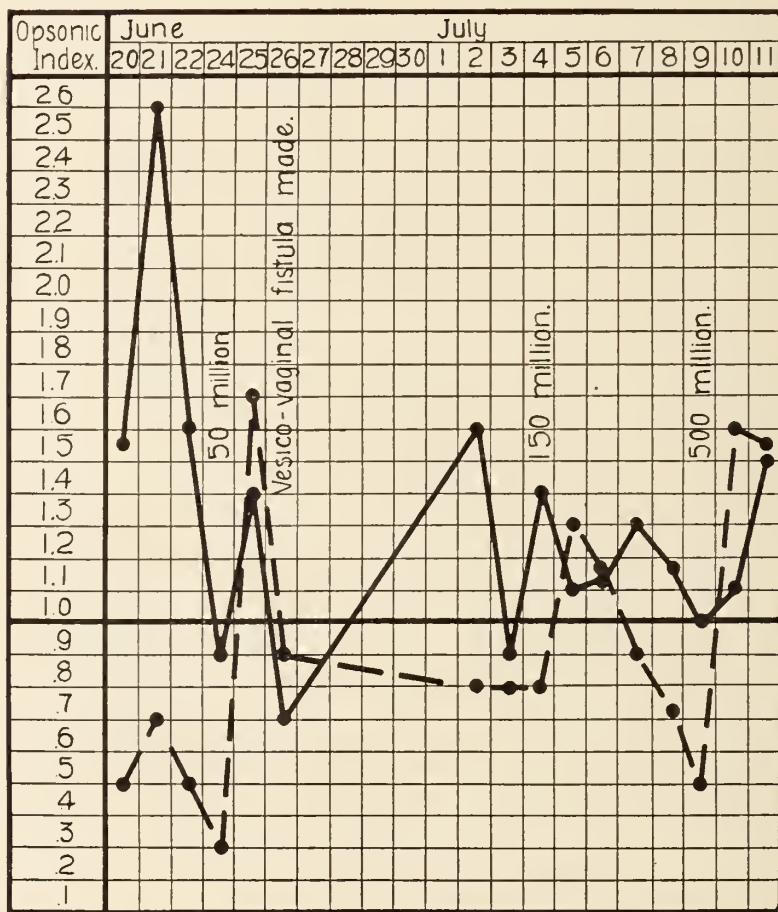


CHART 1.—Comparison of the opsonic index obtained with normal and patient's leukocytes. Solid line=Opsonic index with normal leukocytes. Broken line=Opsonic index with patient's leukocytes.

and 3) we get an idea of the phagocytic power of the patient's leukocytes as compared with normal leukocytes under the influence of normal serum. The solid line in Chart 2 gives a number of results obtained in this way and it appears that under the influence of normal serum the patient's leukocytes became less active after the second and



third injections, slightly more active after the first injection. In the same way by dividing the result obtained with the patient's leukocytes and serum by the result obtained with normal leukocytes and

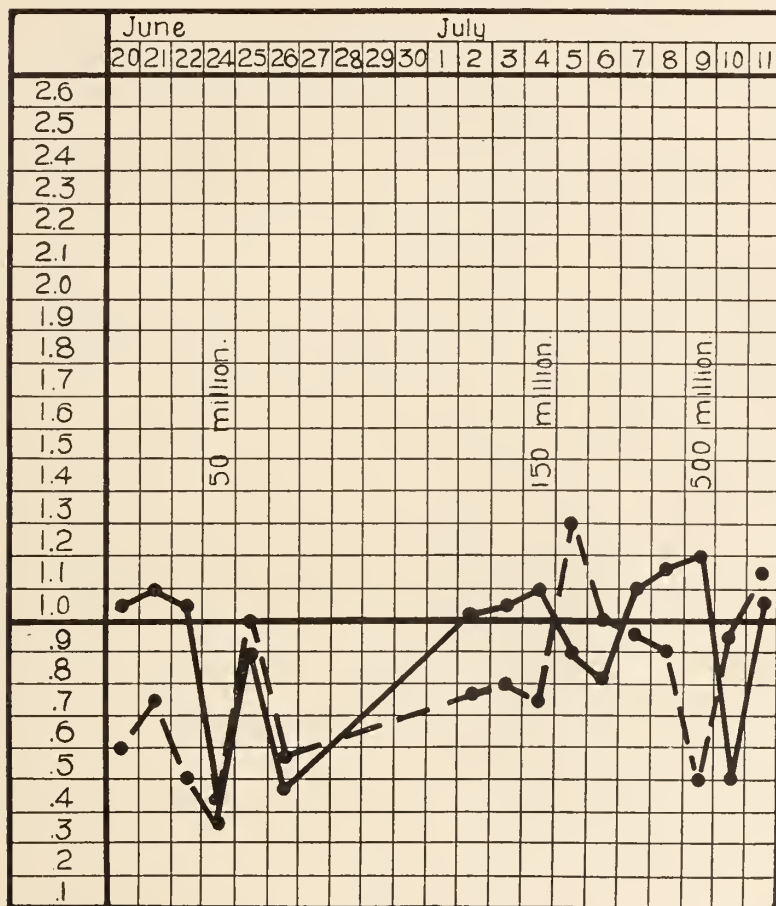


CHART 2.—Comparison of the phagocytic power of normal and patient's leukocytes. Solid line=Phagocytic power of patient's leukocytes in normal serum. Broken line=Phagocytic power of patient's leukocytes in patient's serum. (Standard of comparison—the heavy line at 1.0—is formed by the phagocytosis obtained in mixtures containing normal serum and normal leukocytes.)

serum we obtain an idea of the phagocytic power of the patient's leukocytes as compared with normal leukocytes under the influence of the patient's serum (Lines 2 and 4). All three injections appear

to have been succeeded by an increased phagocytic power of the patient's leukocytes when in patient's serum (broken line, Chart 2). This reaction was specific for the bacillus injected and not obtained with pneumococci or tubercle bacilli.

To summarize, the injections were associated with (1) a rise in the opsonic power of the serum for normal leukocytes in two instances when the index was normal on the day of injection, and a drop once when the opsonic index was 1.4 at the time of injection; (2) a uniform rise in the opsonic power of the patient's serum for the patient's leukocytes; (3) a drop in the activity of the patient's leukocytes in normal serum on two occasions and a slight rise on one; (4) a uniform increase in the activity of the patient's leukocytes in the patient's serum. It is of interest to note that the injections were followed by a moderate leukocytosis. It is not unlikely that in many chronic infections the same conditions prevail as in this case, viz., that while the opsonic index in the usual sense may be quite high, yet the phagocytic power of the patient's blood (leukocytes and serum) may be below normal to the particular microbe causing the infection. In Table 2 it is noted that there occurred moderate agglutination of bacilli in the presence of the patient's serum. This was constant both before and after the injections, and the agglutination was not more marked when the index was 0.3 than when it was 1.7. Dilution experiments also gave the same results, distinct agglutination occurring at the end of eight hours in a dilution of 1:250 in the patient's serum, while in the normal serum discernible agglutination disappeared in dilutions above 1:10.

The opsonic and agglutinating power of the serum on the colon bacillus isolated from the bladder seemed to be normal.

#### SUMMARY.

The diphtheritic ulceration of the bladder in this case was caused by the pseudodiphtheria bacillus for the following reasons:

1. The constant presence of the bacillus in the urine and its predominance in the smears from the scrapings.
2. The specific and peculiar behavior of the blood toward this bacillus especially after the injection of dead bacteria with apparent benefit for the patient.

3. The high agglutinating power of the serum on this bacillus and absence of such power on the colon bacillus.

While the injection of killed bacteria tended to raise the power of the serum for normal leukocytes, the rise was uniformly greater for the patient's leukocytes and this seemed specific with respect to the bacterium injected and presumably the cause of the infection. The phagocytic activity of the patient's leukocytes in the presence of normal serum tended to become less after the injection, but greater when in their own serum, and this was also specific with respect to the bacillus injected. This observation suggests that too much reliance should not be placed on the opsonic index as determined in the usual way as a criterion of the actual conditions and that determination of the specific phagocytic power of the patient's blood would be of greater value because it approximates more closely actual conditions.

## A STUDY OF BACILLUS BULGARICUS.\*

(Synonyms: *Bacillus* of Massol, *B. acidophilus*, *Boas-Oppler bacillus*, *Bacillus panis fermentati*, *Streptobacillus lebenis*, *Leptothrix buccalis*.)

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IN a preliminary note, presented at the meeting of The Society of American Bacteriologists at Baltimore, 1908, we reported the finding of a bacillus resembling *B. bulgaricus*, an organism which has been studied extensively because of the fact that Metchnikoff recommends its use in the artificial preparation of buttermilk. According to our investigations, this bacillus is the principal agent which produces lactic acid in salt-rising bread, the acid thus formed decomposing sodium bicarbonate, with the liberation of carbon dioxide. Salt-rising bread, as is well known, is started by a mixture of meal, usually cornmeal, with milk, saleratus, and a small amount of salt. The liberation of carbon dioxide causes the rising of the dough. We ascertained that the active bacillus was present in the cornmeal, since a mixture of cornmeal and sterilized milk revealed the organism in large numbers and practically in pure culture. This bacillus is somewhat difficult to cultivate, and we found that the ordinary laboratory media are quite inadequate. Sterilized milk or media prepared from milk seem the most favorable, although the addition of glucose to broth increases multiplication if the incubation temperature is high enough, i. e., 37° C. or higher.

Several descriptions of bacilli producing a large amount of acid, much more than the ordinary lactic-acid bacteria, have appeared in the literature, and the suggestion of Metchnikoff has induced several workers to take up the study again. Fermented milk beverages, especially, have been investigated, and organisms found which resemble each other closely. They are described as large bacilli, which occur singly or in filaments, produce large amounts of acid in milk (up to three per cent), and are gram positive. The descriptions are not complete, owing probably to the fact that the organisms do not

\* Received for publication April 10, 1909.

grow readily on ordinary media; but a comparison of the reports of various authors gives the impression that the bacilli resemble each other, and may be identical. One class of these bacteria, producing large amounts of acid, is described under the collective name acidophile, a term which is misleading since the bacilli do not prefer an acid medium but are able to multiply more rapidly in the presence of a higher percentage of acid than are most other bacteria. As a matter of fact, neutral media are more suitable for the growth of these bacteria than acid media. In some textbooks we find an organism mentioned under the name *Leptothrix buccalis*. The species is not well defined and probably covers several kinds of bacteria. It occurs in saliva, forms acid in the mouth, and is considered the cause of caries of the teeth. To judge from the meager descriptions, this *Leptothrix* should be classed with acidophile bacteria.

Emmerling<sup>1</sup> saw a long bacillus in the Armenian fermented milk "mazun," but he was unable to cultivate it. He also found a small bacillus forming dextro-rotatory lactic acid "which apparently is identical with Hueppe's *B. acidi lactici* and has been thoroughly studied by Günther and Thierfelder." Freudenreich<sup>2</sup> studied "kefir" and described similar acid-forming bacilli. Finkelstein<sup>3</sup> found a branching, filament-forming microorganism in the intestinal canal of bottle-fed and breast-fed infants. He isolated the organism by using broth with the addition of 0.5 per cent acetic acid and two per cent glucose. Moro<sup>4</sup> found a similar organism by using beerwort as a culture medium. The colonies are described as having branching edges. Rodella<sup>5</sup> described the colonies of a similar bacillus as resembling a ball of twine. The organism grows at high temperature only and not on ordinary media. Milk or broth with two per cent glucose is a favorable medium. From liquid media the bacilli are generally single, but sometimes long threads appear. Henneberg<sup>6</sup> gave account of a similar organism under the name *B. panis fermentati*. Grigoroff,<sup>7</sup> in Massol's laboratory, examined "yoghurt," the fermented milk of Bulgaria, and the organism he found was named *B. bulgaricus* or the bacillus of Massol. Cohendy<sup>8</sup> and other authors, besides studying *B. bulgaricus*, made experiments in regard to the possibility of acclimatizing it in the digestive tract of man. Bertrand and Weissweiler<sup>9</sup> tested the same organism as to the chemical changes brought about in milk. They conclude that the casein and butterfat are decomposed to some extent, and that the acid produced is principally lactic acid, with small amounts of volatil acids. The lactic acid is a mixture of dextro-rotatory and levo-rotatory, the former predominating. Rist and Khouri<sup>10</sup> made an extensive study of the Egyptian fermented milk "leben" and called the principal acid-forming organism *Streptobacillus lebenis*. Mereschewsky<sup>11</sup> distinguished

<sup>1</sup> *Centralbl. f. Bakt.*, Abt. 2, 1898, 4, p. 418.

<sup>2</sup> *Ann. de l'Inst. Pasteur*, 1895, 9, p. 811.

<sup>3</sup> *Deutsch. med. Wchnschr.*, 1900, 26, p. 263.

<sup>4</sup> *Wiener klin. Wchnschr.*, 1900, 13, p. 114.

<sup>5</sup> *Centralbl. f. Bakt.*, Abt. 1, 1901, 29, p. 717.

<sup>6</sup> *Ibid.* Abt. 2, 1903-4, 11, p. 168.

<sup>7</sup> *Rev. méd. de la Suisse Rom.*, Genève, 1905, 25, p. 714.

<sup>8</sup> *Compt. rend. de la soc. biol.*, 1906, 60, p. 364.

<sup>9</sup> *Ann. de l'Inst. Pasteur*, 1906, 20, p. 977.

<sup>10</sup> *Ibid.*, 1902, 16, p. 65.

<sup>11</sup> *Centralbl. f. Bakt.*, Abt. 1, 1905, 30, p. 380.



two strains of the so-called *B. acidophilus* on the strength of finding differences in the colony-formation. The Sardinian fermented milk "gioddù" was examined by Grixoni<sup>1</sup> who found a bacillus which occurs in pairs or chains of six to eight members, stains at the poles or exhibits granules and does not grow on the ordinary laboratory media. Weigmann, Gruber, and Huss<sup>2</sup> described a bacillus which produces large amounts of lactic acid. Dügge<sup>3</sup> found in "mazun" prepared from buffalo milk, sheep's milk, or goat's milk, a long bacillus which forms flocculent ("flöckige") colonies, grows well at 30° C. or more, but not at 20°. The amount of acid produced was 2.89 per cent. Piorkowski<sup>4</sup> examined "yoghurt" and found a large bacillus, which resembles the hay bacillus morphologically. The colonies have branches or uneven edges like a cactus plant. The optimum temperature was found to be 45° C. Sewerin<sup>5</sup> called attention to the identity of the Bulgarian bacillus and the *Streptobacillus lebanis* of Rist and Khouri. He studied two varieties, one forming a slimy milk, the other not; otherwise the two strains were identical. He also stated that the slime-producing variety loses this property after some time. The amount of acid produced by the slimy variety was 2.37 per cent after seven days; by the other variety 1.64. The curd forms slowly, more so than with *Strept. lacticus*. *B. bulgaricus* loses its vitality rapidly.

A bacillus which seems to resemble the organisms described by the authors mentioned was found by Boas and Oppler<sup>6</sup> in the gastric fluid of patients with carcinoma of stomach. This organism has never been studied thoroughly, chiefly because it is difficult to cultivate by ordinary methods. Morphologically it is quite similar to the acid-producing bacilli of other authors, showing granules and vacuoles in stained preparations. It occurs in filaments, and produces large amounts of lactic acid. Strauss<sup>7</sup> found the same bacilli in small numbers in normal gastric fluid. In stains from the stomach the bacilli have been described as staining solid with carbol fuchsin and granular with methylene blue. The granules were sometimes quite large, and the unstained portions might be mistaken for spores. The gram stain is positive. The colonies in a medium made by adding agar to gastric juice had the shape of leaves with thornlike protuberances. Milk was coagulated in one to three days. Schlesinger and Kaufmann<sup>8</sup> found this organism in 19 cases of carcinoma of the stomach out of 20 examined. The same authors cultivated the organism on meat-peptone agar with the addition of glucose, and in beerwort. Sternberg<sup>9</sup> described the colonies of the Boas-Oppler bacillus as minute points with woolly edges. The bacilli are straight or some slightly curved. They stain with the anilin dyes, and sometimes vacuoles appear, resembling spores. If the colonies are numerous, they give the plate the appearance of being covered with dust. On meat-peptone agar they grow sparingly and not below 37° C. They grow well in broth with sugars. Flagella were stained by Van Ermengen's method. Milk is coagulated after two to three days, but the property to coagulate is easily lost. Sandberg<sup>10</sup> gave a complete list of the literature and made a careful study of the organism. He found two types of colonies, one with woolly edges and small branches, the other more solid. The bacilli resist a high degree of acidity and are longer and larger in acid media than when the reaction is neutral.

<sup>1</sup> *Centralbl. f. Bakt.*, Abt. 2, 1905-6, 15, p. 750.

<sup>2</sup> *Ibid.*, Abt. 2, 1907, 19, p. 70.

<sup>3</sup> *Ibid.*, Abt. 2, 1905-6, 15, p. 577.

<sup>4</sup> *Ibid.*, Abt. 2, 1908, 21, p. 95.

<sup>5</sup> *Ibid.*, Abt. 2, 1908, 22, p. 3.

<sup>6</sup> *Deutsch. med. Wchnschr.*, 1895, 21, p. 73.

<sup>7</sup> *Ztschr. f. klin. Med.*, 1895, 28, p. 578.

<sup>8</sup> *Wien. klin. Rundschau*, 1895, 9, p. 225.

<sup>9</sup> *Wien. klin. Wchnschr.*, 1898, 11, p. 744.

<sup>10</sup> *Ztschr. f. klin. Med.*, 1903-4, 51, p. 80

The author claims slight motility for his organism. Rodella<sup>1</sup> asserted identity of the so-called acidophile bacteria, the Boas-Oppler bacillus, *B. bifidus* of Tissier, and the other bacilli forming large amounts of lactic acid. Kuntze,<sup>2</sup> in an exhaustive treatise, described two bacilli which differ slightly. The one shows many granules ("Körnchen Bacillus") when stained with Neisser's stain or with alkaline methylene blue, the other (*B. bulgaricus*) stains more solidly. The first bacillus grows at a lower temperature than the other. Both grow poorly on the ordinary laboratory media, but more luxuriantly on media with the addition of glucose. The colonies are like snowflakes and sometimes cloudy and dim. The author found the "körnchen" bacillus in the stomachs of calves, and believes that "yoghurt" was originally prepared by adding the stomach contents of calves to boiled milk. The filament formation is more pronounced in cultures of the "körnchen" bacillus than in those of the Bulgarian bacillus. The colonies of the former have rootlike branching; the latter are more compact. The author admits that the two organisms are probably identical. Ficker<sup>3</sup> found similar bacilli in smears from the esophagus of man and in human feces. With Lugol's solution and gentian violet these bacilli stain deeply blue or violet. They form filaments and stain solidly from stools, but show granules when stained from the esophagus. The author ascribed this difference in staining to the presence of starch in the esophagus and its absence from feces. The bacilli produce large amounts of lactic acid. They multiply in cases of carcinoma of the stomach because of the absence of hydrochloric acid, but in normal stomachs the presence of acid restrains their growth. They do not form spores and are not motile. They were found in nearly all of 148 cases examined, when the normal amount or even an excessive amount of acid was present. They are facultative anaerobes. They produce lactic acid in the stomach and in other parts of the digestive tract. Hastings,<sup>4</sup> in a short note, reported the occurrence of large bacilli producing a great amount of acid in milk soured at high temperature for several weeks.

A survey of the descriptions of the various authors quoted shows clearly that these organisms have not been studied thoroughly and under uniform and varied conditions. It is difficult, therefore, to form an opinion as to the identity of all the different forms mentioned. The differences, however, are insignificant. A few observers have described motility of the bacillus, but most have not. Otherwise the variations, such as that of colony-formation and staining properties in relation to the appearance of granules under different conditions, are not of sufficient weight to separate species. We are really dealing with bacteria of the following characteristics: They are fairly large bacilli, the length of which varies according to the composition and reaction of the culture medium. In neutral media the forms are shorter than in media containing considerable acid. The bacilli occur singly or form filaments of varying length, and often the filaments are so numer-

<sup>1</sup> *Centrabl. f. Bakt.*, 1908, 47, p. 445.

<sup>2</sup> *Arch. f. Verdauungskrankheiten*, 1908, 14, p. 537.

<sup>3</sup> *Ibid.*, Abt. 2, 1908, 21, p. 737.

<sup>4</sup> *Science*, 1908, 28, N. S., p. 656.

ous as to appear under the microscope like a streptothrix. The form of the letter "Y" is frequently observed. The ends of the bacilli are round or square. They stain readily with the anilin dyes, and under some conditions granules appear or the poles only are stained. Young cultures are positive to the gram stain, the result being a beautiful dark blue or violet. In older cultures the gram stain is often negative; or some bacilli stain positive, others negative, so that if dilute carbol-fuchsin is used as a counter stain, a part of a filament appears blue, another part red. The colonies are either solid or have woolly edges or branches. This variation is probably due, at least in part, to the composition and consistency of the culture medium. All observers agree that the colonies are very small, usually minute points. The appearance of the plates has been described as though sand or dust had been mixed with the medium. The bacilli do not grow on the ordinary laboratory media at room temperature, and sparingly at 37° C. They grow well in milk, in media prepared from milk, or if glucose is added to the medium. The most favorable reaction of the culture medium is neutral or slightly acid to phenol phthalein, but growth occurs in the presence of considerable amounts of acid, and vitality is retained in acid media when other bacteria are unable to live or multiply. The optimum temperature seems to be 40° C., some observers reporting 42 to 44°, others even 45°. Milk is coagulated, but somewhat more slowly than by the ordinary lactic-acid bacteria. Large amounts of lactic acid, up to three per cent, are produced from carbohydrates. These bacteria have been found in the esophagus, in the stomach of man and calves, in the feces of man, and in the fermented milks of the Orient.

From these considerations it seems reasonable to suppose that one organism has been described by many authors under different names. According to the source from which it was obtained the characteristics have been found to vary somewhat, not sufficiently, however, to justify the recognition of different species. The difficulty of cultivating the organism is no doubt largely responsible for the fact that it has been rarely found, and then only when conditions were exceptionally favorable for its growth, as in fermented milks. By employing suitable media and methods of isolation we have succeeded in cultivating the bacillus from a variety of sources. The medium

used was ordinary broth with the addition of 0.5 per cent glacial acetic acid and two per cent glucose. The large amount of acid restrained most other bacteria, while injuring *B. bulgaricus* but little. By these methods we succeeded in isolating cultures of the bacillus from the following material:

1. In all of 8 samples of cow feces.
2. In all of 6 samples of horse feces.
3. In all of 6 samples of human feces.
4. In 4 of 5 samples of human saliva. In 3 of these it was very numerous.
5. In 5 samples of soil where manure of cows or horses might be expected to be present.
6. In 1 out of 2 samples of soil where the presence of manure was excluded.
7. In 6 samples of market milk kept at 37° or 42° C. for several days.
8. In fodder for cattle, viz., bran, silage, and dry brewers' grains.
9. In the following foods and spices: cornmeal, sauerkraut, olive juice (from bottled olives), dill pickles, pepper mango.
10. In small numbers in 2 samples of normal gastric fluid and in exceedingly large numbers in gastric fluid from 2 cases of carcinoma of the stomach.

We did not succeed in isolating the bacillus from wheat flour, roquefort cheese, picalilli, sweet and sour pickles.

We judge from the various sources from which the bacilli were isolated that excessive acid or the absence of carbohydrates constitute unfavorable conditions for the life of the organism, and that if they gain access to substances under such conditions they do not multiply, but gradually die.

Of the seven samples of soil examined for the presence of *B. bulgaricus* we found that in earth from a paddock, where horses and cows were kept, the bacillus was present in large numbers. Soil in front of a horse stable and from the bank of a small creek contained it in fairly large numbers. In one sample obtained from the roadside where the soil had been cut recently for road improvement we found the bacillus in small numbers, and in samples from the shore of a small inland lake as well as in soil taken from beneath the roots of grass under a clump of trees we were unable to find any bacilli. In garden soil we had no trouble finding bacilli in fairly large numbers. It seems probable, therefore, that *B. bulgaricus* occurs naturally in soil, but in large numbers only when manure is present.

#### METHODS OF STUDY AND ISOLATION.

We found the following method successful for isolating the Bulgarian bacillus. The material to be examined was added to a tube of litmus milk and incubated at 42° C.

for 24 to 48 hours. Feces or other material containing large numbers of bacteria was added to acid broth composed of ordinary meat infusion or meat-extract broth with addition of 0.5 per cent glacial acetic acid and two per cent glucose. A transfer to litmus milk was made after 24 hours. The large amount of acid seems to restrain other bacteria from multiplying and interfering with the growth of *B. bulgaricus*. The litmus milk tubes were then used for plating in milk-serum agar, to which we also added 0.5 per cent glacial acetic acid.<sup>1</sup> The plates were incubated at 42° C. Microscopic colonies are usually visible in 24 hours, and are discernible to the naked eye after 48 hours. They are small and resemble colonies of streptococci, but may be distinguished from these under the hand lens by their slight irregularity of contour. Un-

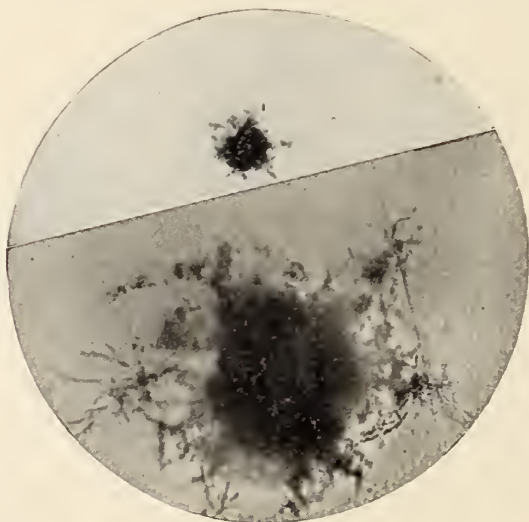


FIG. 1.—*B. bulgaricus* from normal mouth, 48-hour colonies on acid whey agar at 42° C. Small colony  $\times 40$ . Large colony  $\times 220$ .

der the No. 3 objective they have the characteristic woolly appearance of *B. bulgaricus*. If present in the original material, they occur on such a plate in nearly pure culture, other forms having been suppressed by the high temperature and by the acidity of the agar. The litmus milk tubes themselves are, after 48 hours, an indication of the presence of *B. bulgaricus* in the material added to them. The color of the litmus caused by *Strept. lacticus* is a light pink, but that caused by *B. bulgaricus* soon becomes a deep vivid pink, which is quite distinctive. We were soon able to tell at a glance from a series of milk tubes which were likely to prove positive for this organism.

**Cultural characters.**—On whey-agar (one per cent acid to phenolphthalein) plates at 37° to 42° C. the 24-hour colonies are minute and almost indistinguishable to the eye from streptococcus colonies. If numerous they show on the plate like fine grains

<sup>1</sup> Preparation of milk-serum agar: Boiling certified milk is coagulated by addition of a few drops of acetic acid. The serum is filtered, the reaction made 1 per cent acid to phenolphthalein, 1 per cent Witte's peptone and 2 per cent glucose added, and after solution 1.5 per cent agar is dissolved in it. The whole is then clarified with the whites of eggs or dry egg albumin.



of sand. Under the microscope the edges of even the smallest colonies are seen to be broken or fringed and the 48-hour colonies are characteristically densely matted, woolly at the center and branching at the edges (Fig. 1).<sup>1</sup> The colonies remain small and are mostly under the surface. On acetic-acid agar they appear readily and numerous, but remain minute and are smaller than streptococcus colonies. On plates from the gastric juice of a case of carcinoma two somewhat different types could be distinguished, one being the same as usual, the other more dense.

On whey-agar slants the cultures show a good development both on the surface and in stabs. The surface growth consists of a large number of small colonies, often more or less confluent so as to form a delicate veil. The stab is fairly vigorous and granular.

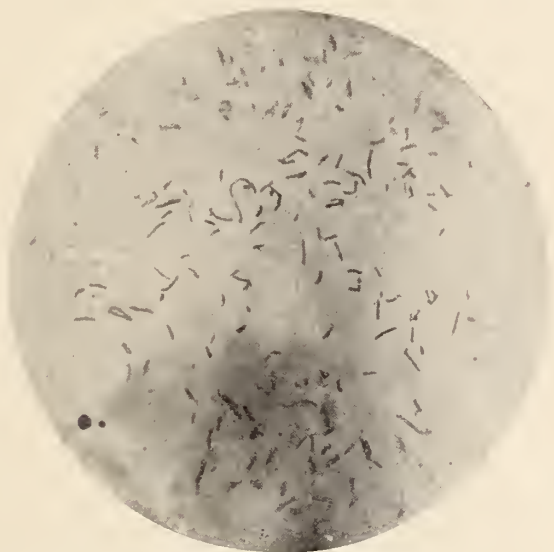


FIG. 2.—*B. bulgaricus* from milk. Pure culture on whey agar.  $\times 300$ .

Milk, as mentioned above, is the most favorable medium. It is coagulated more slowly than by *Strept. lacticus*, usually not before 48 or 72 hours at 37° C., often longer. Coagulation takes place more rapidly at 42° than at 37°, and the power of acid-production is increased somewhat by reinoculation from tube to tube of milk at 42° as soon as the curd is formed, i. e., the interval shortens. The coagulum is soft, smooth, and easily broken up. Whey is rarely separated. Some strains form sliminess of the milk, but this power is readily lost. Milk cultures have a peculiar cheesy butyric-acid odor and the taste after about 10 days is extremely acid and acrid.

Ordinary laboratory media, such as plain agar, broth, potato, or gelatin, allow little or no development. Some growth occasionally takes place on glucose agar. No gas is formed on glucose media. Vitality of cultures decreases rapidly on any medium except milk. Even a whey-agar culture soon dies out.

There is little or no growth below 37° C. The optimum temperature appears to be

<sup>1</sup>The photographs in this paper were made by Mr. A. C. Hicks of this laboratory to whom our acknowledgments are due.



FIG. 3.—*B. bulgaricus* from milk. Pure culture on whey agar showing branched bacillus. Gram-Weigert stain.  $\times 1,100$ .

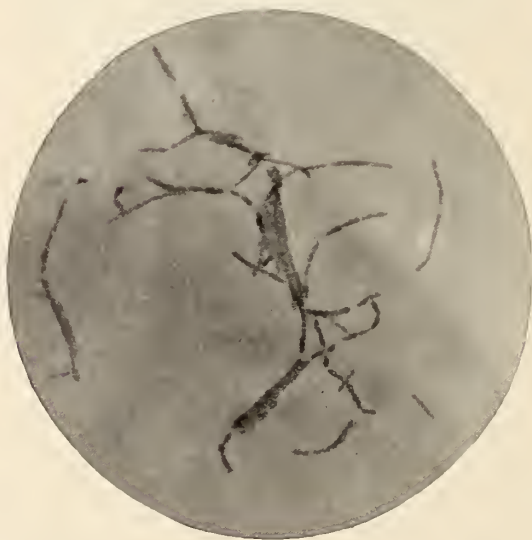


FIG. 4.—*B. bulgaricus* from normal human feces. Pure culture on whey agar. Gram-Weigert.  $\times 1,100$ .

about 42° to 44°. By growing cultures in a hydrogen atmosphere in Novy jars we found that the organism grows anaerobically as well as aerobically.

**Morphology.**—The bacilli are large and usually have square ends like *B. anthracis* (Fig. 2). They are non-motile and non-sporing in culture. They often occur in chains (streptobacilli) and in milk grow out in long filaments. True dichotomy has been observed in stained preparations, the bacilli assuming the shape of the letter "Y" (Fig. 3). The organism stains readily with all the ordinary dyes and deeply blue or violet by Gram's method,<sup>1</sup> especially in young cultures. With alkaline methylene

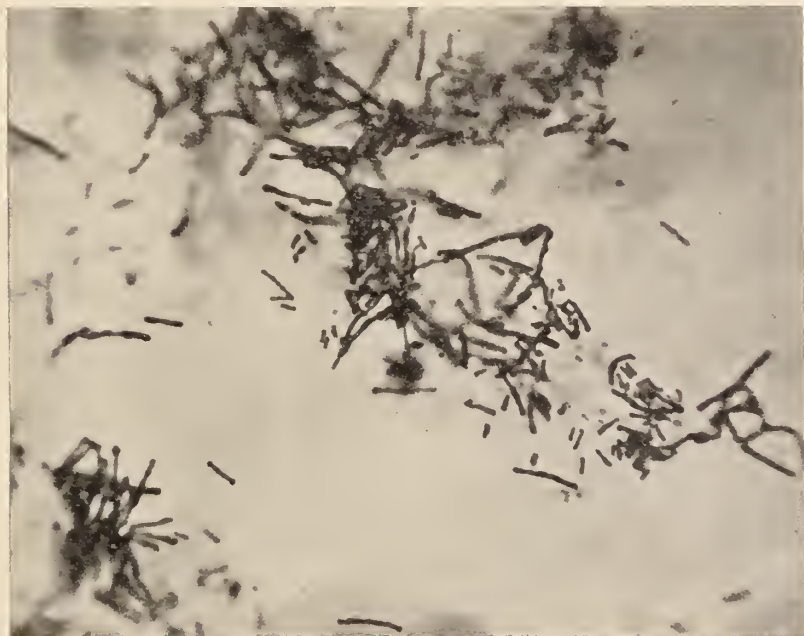


FIG. 5.—Smear from gastric fluid of a case of carcinoma of the stomach. Large and small bacilli showing polar granules. Practically pure culture. Gram-Weigert.  $\times 1,800$ .

blue some strains stain solidly (streptobacilli) while others color lightly but show deeply stained round granules. The same strain may show deeply stained granules in milk and stain solidly when grown on agar. A stained preparation made from one colony shows great variation both in length and thickness of the bacilli. From milk cultures the appearance of the organism is very characteristic, consisting of long irregularly stained filaments and single bacilli, both showing unstained portions or vacuoles, and often slightly enlarged ends with unstained nodes between those of adjoining bacilli. In many cases the granules were only indicated by a somewhat darker staining at the ends of the cell. Curved and warped organisms are sometimes seen as well as tangled

<sup>1</sup> Both smears and culture preparations are best stained by the Weigert modification of Gram's stain (decolorization in anilin-xytol and washing in xytol). The bacilli in a loopful of milk culture are difficult to demonstrate by other means, but show clearly, especially the granules, by this method.

masses of threads. In a smear made directly from the gastric juice of a case of carcinoma a great variety of forms could be seen. Short forms with rounded ends and beautiful granules were present, as well as long, large forms showing both distinct granules and darker staining portions. The long, solidly staining form was most prevalent, and long threads were observed. The organisms were exceedingly numerous in this preparation (Fig. 5).

#### PRODUCTION OF ACID IN MILK.

In order to compare the different strains isolated both with each other and with recognized strains of *B. bulgaricus* obtained from other laboratories, we examined a series carefully with respect to the power of acid-production in milk. Flasks containing 300 c.c. of sterilized milk each were inoculated by pouring into them 5 c.c. each of 48-hour-old milk cultures of the different strains. One set of flasks was also inoculated with a mixture of *B. bulgaricus* and a strain of *Strept. lacticus* of the same age and grown at 37°. The flasks were incubated at 37°, 5 c.c. being removed from each at certain intervals by means of sterile pipettes, and the acidity determined by titration at boiling temperature with  $\frac{1}{20}$  normal NaOH and phenolphthalein as indicator. The amount of acid in the following table is expressed in terms of lactic acid.

TABLE 1.  
ACID-PRODUCTION IN MILK BY *B. bulgaricus* FROM DIFFERENT SOURCES.

SOURCE	ACIDITY IN TERMS OF LACTIC ACID AFTER								
	1 Day	2 Days	3 Days	6 Days	10 Days	17 Days	23 Days	31 Days	42 Days
Cow feces.....	0.10	0.34	0.52	0.05	1.25	1.44	1.68	1.68	1.70
" ".....	0.22	0.62	1.28	1.03	2.48	2.73	2.72	2.73	2.73
" ".....	0.18	0.20	0.20	0.88	1.03	1.15	1.25	1.40	2.12
" ".....	0.25	0.30	0.30	0.00	1.30	1.54	1.54	1.53	1.53
" ".....	0.10	0.20	0.30	0.85	1.33	1.51	1.54	1.52	1.52
" ".....	0.17	0.20	0.22	0.35	0.56	0.68	0.85	0.80	0.93
" ".....	0.15	0.20	0.24	0.26	0.28	1.25	1.70	1.84	1.98
Horse.....	0.17	0.20	0.40	0.81	1.01	1.27	1.40	1.52	1.52
" ".....	0.18	0.34	0.57	1.27	1.71	1.97	2.05	2.10	2.10
" ".....	0.18	0.25	0.40	0.75	0.87	0.00	0.04	1.44	1.83
" ".....	0.18	0.67	1.22	1.03	1.70	1.70	1.78	.....	.....
Human feces...	0.22	0.40	0.58	0.82	1.21	1.30	1.36	1.36	.....
" ".....	0.17	0.28	0.48	0.00	1.13	1.16	1.44	1.55	1.71
Milk.....	0.21	0.73	1.13	1.87	2.17	2.44	2.49	2.65	2.85
" ".....	0.16	0.16	0.17	0.20	0.20	0.21	0.27	0.32	0.38
Cow skin.....	0.18	0.22	0.24	0.36	0.54	0.83	1.08	1.07	.....
Brewers' grains	0.18	0.38	0.87	1.78	2.15	2.10	2.17	.....	.....
Bran.....	0.16	0.18	0.30	0.85	1.00	1.13	1.25	1.34	1.40
Cornmeal.....	0.16	0.10	0.26	0.54	1.23	1.45	1.75	1.00	1.00
Acid saliva.....	0.16	0.17	0.10	0.45	0.82	0.95	1.12	1.21	1.35
Nor. ".....	0.14	0.32	0.80	1.12	1.30	1.42	1.55	1.50	1.50
Olives.....	0.14	0.15	0.10	0.22	0.20	0.32	0.30	0.70	1.23
Sauerkraut.....	0.17	0.22	0.24	0.54	0.88	1.00	1.01	1.00	1.00
Dill pickles.....	0.14	0.65	0.77	0.82	0.85	0.84	.....	.....	.....
<i>Strept. lacticus</i> ..	0.20	0.53	0.60	0.73	0.73	0.73	.....	.....	.....

The table shows clearly that the amount of acid produced by

different strains varies within wide limits. We are unable at present to account satisfactorily for this variability. The highest amount was produced by a culture sent to us from the laboratory of Mr. Simeon Keith, in Boston. One strain isolated from milk came very near producing the same amount. More than two per cent were produced by strains isolated from brewers' grains, cow feces, and horse feces. The normal temperatures of cows and horses are somewhat higher than of man, and this suggests the possibility that the vitality of the Bulgarian bacillus is preserved under such temperature conditions. It is not surprising that a large amount of acid is produced from a milk strain, since milk is the most favorable medium for the growth of the Bulgarian bacillus. Dry brewers' grains possibly furnish favorable conditions for preserving the vitality, on account of the presence of considerable amounts of carbohydrates. No relation between acid-formation and slime-production was observed. In mixed cultures (*B. bulgaricus* and *Strept. lacticus*) the amounts of acid were generally smaller than in pure cultures, but the initial acid-formation was more rapid and the milk coagulated earlier (Chart 1.).

The percentage of volatil acids was determined in several cultures, including the one obtained from Mr. Keith, by passing steam through 100 gm. of the sour milk and collecting the distillate in a definite amount of  $\frac{1}{20}$  normal NaOH. This process was continued until no more acid passed over, and the excess of NaOH was found by titration with  $\frac{1}{20}$  normal HCl. Thus we determined that the amount of volatil acid formed was 5.8 to 6.1 per cent of the total amount produced. The remaining acid was taken to be lactic acid. The optical activity of the lactic acid was determined after preparing the zinc salts in the usual manner. Solutions of these zinc salts were optically inactive and the mother liquor obtained after removing the salts was found to be inactive also. Therefore the lactic acid produced by *B. bulgaricus* is inactive without a trace of active acid.

The Bulgarian bacillus decomposed the casein and the butterfat to some extent. We have not determined the exact nature of this process, but the odor of butyric acid develops after several days, and the taste, besides being extremely sour, is somewhat bitter, perhaps due to the formation of caseones.



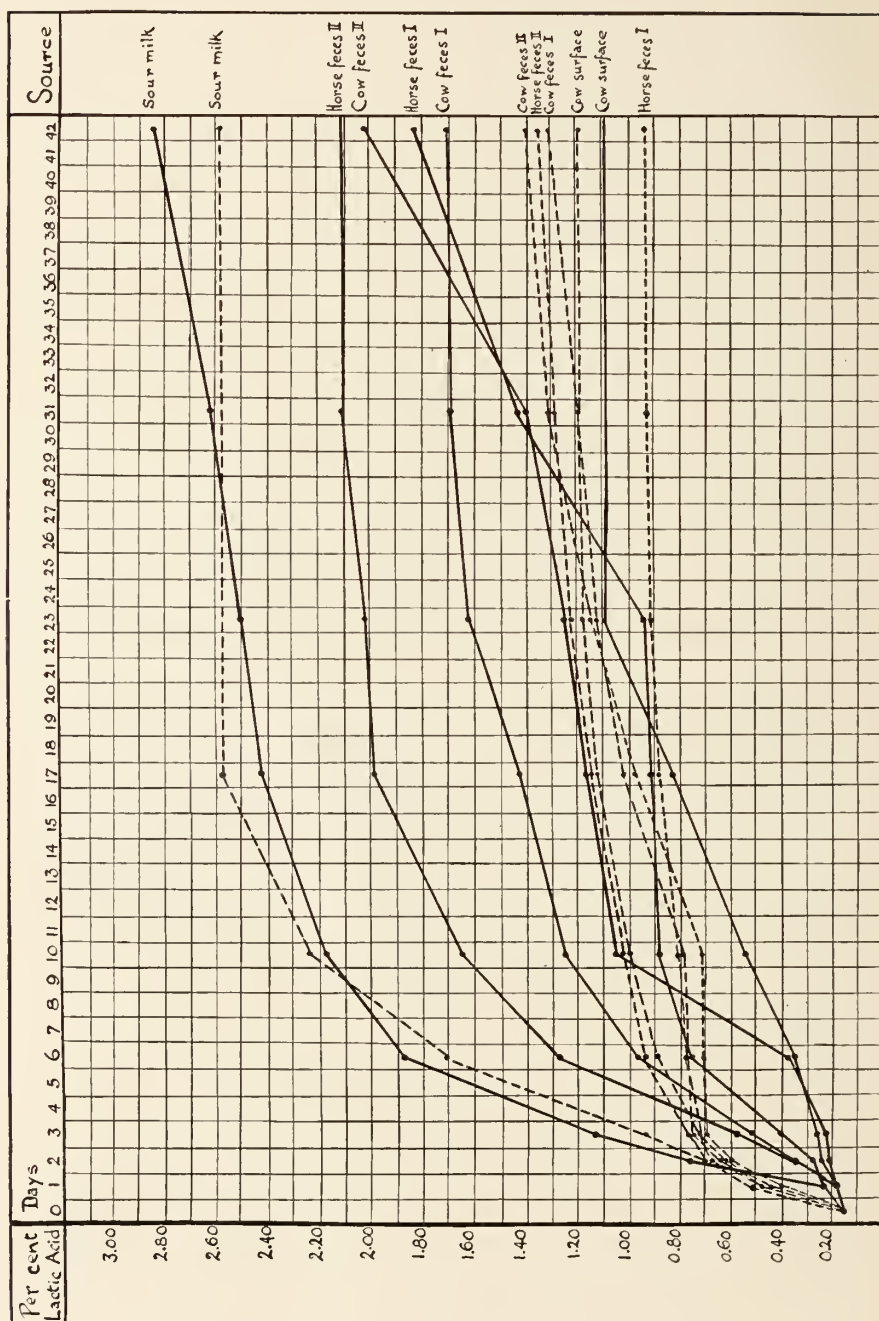


CHART 1.—Production of lactic acid in milk at 37°C. Solid line=*B. bulgaricus*; broken line=*Strept. lacticus*.

The presence of *B. bulgaricus* in the apparently normal mouth, stomach, and feces of all but one case examined is a fact of considerable interest in view of the attempts which have been made to establish this organism in the intestinal tract by feeding.<sup>1</sup> Considering the widespread distribution which our methods of isolation have shown, there is little doubt that the lactic-acid bacillus is constantly being ingested with milk and other foods and is constantly present in the intestinal tract in some numbers, varying with the amount of normal acidity or other conditions. The number of colonies found on our plates after identical methods of enrichment and incubation differed greatly with respect to saliva from different individuals, as did also the seeming degree of vigor of the culture obtained. It is possible that the presence in the mouth of large numbers of *B. bulgaricus*, because of its lactic-acid-producing properties, may be a cause of caries of the teeth. Smears from normal stomach contents as well as from some samples of saliva often failed to show any gram-positive bacilli, but by the selective method described above we had no difficulty in final demonstration. The ingestion of milk containing large quantities of *B. bulgaricus* serves then only to increase the numbers of an organism already an ordinary habitant of the intestinal tract, and its presence after such a course of feeding is not necessarily a proof of its permanent acclimatization there in large numbers.

#### CONCLUSIONS.

1. *Bacillus bulgaricus* is widely distributed in nature and may be isolated by means of incubation in milk at high temperature and subsequent plating on a medium containing 0.5 per cent glacial acetic acid and two per cent glucose.

2. *B. bulgaricus* occurs normally in human feces, in the feces of cows and horses, also in a variety of sour and aromatic foods, in food for cattle, in normal saliva, in normal gastric juice and in gastric juice when hydrochloric acid is absent, in various fermented milks, in ordinary market milk, and in soil, both manured and not manured.

3. Some bacilli isolated from various fermented milks, the so-called acidophile bacilli found in the intestinal tract of man, the so-called Boas-Oppler bacillus, *B. panis fermentati*, *Streptobacillus lebenis*,

<sup>1</sup> Herter and Kendall, *Jour. Biol. Chem.*, 1908, 5, p. 293; Cohendy, *Compt. rend. de la soc. biol.*, 1906, 60, p. 364; Metchnikoff, *The Prolongation of Life*, New York, 1908.

an organism sometimes called *Leptothrix buccalis*, and possibly *B. bifidus* of Tissier are identical with *B. bulgaricus*.

4. *B. bulgaricus* grows poorly on the ordinary laboratory media and has therefore probably been frequently overlooked by investigators. Milk or media composed of whey, peptoné, and glucose are most favorable.

5. The optimum temperature for growth is about 42° to 44° C. The Bulgarian bacillus does not grow at ordinary room temperature.

6. *B. bulgaricus* grows as well under anaerobic as under aerobic conditions.

7. The vitality of *B. bulgaricus* is readily lost, especially in competition with other bacteria under ordinary conditions.

8. The amount of acid produced in milk by *B. bulgaricus* may reach more than three per cent. The acid produced is composed approximately of six per cent volatil acid and 94 per cent lactic acid of the optically inactive variety.

9. The casein and fat in milk are partially decomposed by the activity of *B. bulgaricus*.

10. The presence of *B. bulgaricus* in normal saliva may be responsible for caries of the teeth, because of its ability to produce large amounts of acid from carbohydrates.

11. The presence of *B. bulgaricus* in human feces after ingestion of milk fermented by this organism cannot be considered positive proof of its permanent acclimatization in the digestive tract.

## THE BACTERICIDAL SUBSTANCE IN LEUKOCYTES.\*

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WYSSOKOWITCH<sup>1</sup> was the first to call attention to the bactericidal power of the blood of living animals, and to show that non-pathogenic bacteria can be recovered from the organs of the body after their disappearance from the blood. Von Fodor<sup>2</sup> first showed that *B. anthracis* was destroyed by freshly drawn blood. Shortly afterward Nuttall<sup>3</sup> proved that while rabbit's-blood serum destroyed *B. anthracis*, this destroying power was lost on the application of heat or upon standing. Nissen<sup>4</sup> working with rabbit's blood pointed out that only certain organisms were destroyed by it, and that others were only retarded in growth or were entirely unaffected. Thus, *Sp. cholerae*, *B. anthracis*, *B. typhosus*, and the pneumococcus were killed, whereas *Staph. aureus* and *albus*, and *Strept. pyogenes* were only retarded in growth. He further showed that the blood serum was capable of destroying a limited number of organisms only, and that the time required for this destruction varied with the different organisms. Thus, he found that *Sp. cholerae* required 10 to 40 minutes, *B. typhosus*, about two hours for death by blood serum.

Since these investigations were published a vast amount of research has been directed toward elucidating the intimate relations existing between the properties of the blood and the state of natural susceptibility or of resistance to infective processes. Behring,<sup>5</sup> Buchner,<sup>6</sup> Ogata<sup>7</sup>, and many others have obtained remarkable results on the subject, and have given credence to the so-called Buchner's alexin or humoral theory. Metchnikoff and his school,<sup>8</sup> on the contrary, in the face of opposition lasting many years, have offered convincing proofs of the importance of phagocytosis in the protection of the animal body against bacterial invasion. The main theses of the

\* Received for publication March 10, 1900.

<sup>1</sup> *Ztschr. f. Hyg.*, 1886, 1, p. 1.

<sup>3</sup> *Ztschr. f. Hyg.*, 1888, 4, p. 353.

<sup>2</sup> *Deut. med. Wchnschr.*, 1886, 12, p. 617.

<sup>4</sup> *Ibid.*, 1880, 6, p. 487.

<sup>5</sup> *Ibid.*, 1890, 8, p. 412.

<sup>6</sup> *Centralbl. f. Bakt.*, 1889-1890, 5, p. 817; 6, pp. 1, 561; 8, p. 65.

<sup>7</sup> *Ibid.*, 1891, 9, p. 597.

<sup>8</sup> *Immunity in Infective Diseases*, tr., Cambridge, 1905.

Metchnikoff theory are now almost universally accepted, but the exact mechanism of the processes involved is still the subject of keen controversy. For a long time an uncompromising opposition divided the workers in the field of immunity into two parties. Of late, however, a growing tendency is manifesting itself toward a fusion of the rival schools of the humoralists and of the supporters of the phagocytic theory. It is not surprising, however, that on many points, and these not the least important, a bewildering discordance of view still exists. One of the most interesting and vital of these issues relates to the rôle of the leukocytes as the originators of the bactericidal substances in the defensive mechanism of the animal organism. In view of this controversy the following experiments made by the writer may be of interest.

#### METHOD.

The leukocytes used were those of normal rabbits. To obtain polynuclear leukocytes an intrapleural injection of 5 to 10 c.c. of plant-casein emulsion (in 0.85 per cent NaCl solution) was made, and 24 hours later the rabbit was bled to death. The pleural cavities contained a copious turbid exudate. This was pipetted into normal-salt solution with a trace of 1 per cent sodium-citrate solution, was centrifugalized, and then washed in normal-salt solution two or three times. It was then disintegrated in ice-salt mixture for one to two hours, and mixed with an equal bulk of normal-salt solution.

For mononuclear leukocytes an intraperitoneal injection of 5 to 10 c.c. of freshly washed guinea-pig erythrocytes suspended in normal-salt solution containing a trace of sodium citrate was made. From 24 to 48 hours later the rabbit was bled to death and the emulsion treated as above.

The bacteria used were young 18 to 24-hour agar colonies emulsified in broth. The suspension contained 0.05 mg. of bacteria to 0.5 c.c. broth.

In all experiments 0.5 c.c. each of leukocytic and bacterial suspension were mixed, kept at 37° C. 30 minutes, 3 hours, 6 hours, and 24 hours, and then plated in agar.

Inactive suspensions were obtained by heating at 55°-60° C. for 30 minutes.

The controls used were the supernatant cell-free fluid of the exudate, inactivated supernatant cell-free fluid of exudate, fresh rabbit serum, inactivated rabbit serum, and leukocytes disintegrated as above.

Organ extracts were also used, portions of the spleen, liver, bone-marrow of a healthy rabbit being excised immediately after death. These were cut in small pieces and broken up in normal-salt solution by means of a glass rod. The blood was removed so far as possible by repeated washings in fresh saline. The tissue was finally disintegrated in ice-salt mixture for one to two hours as was the leukocytic exudate.

#### I. POLYNUCLEAR LEUKOCYTES.

**Experiment 1.**—A 2,500-gm. rabbit was injected with casein 24 hours previous to bleeding. Polynuclear leukocytes, 85 per cent in the film stained by Giemsa's method. The results of action upon *B. typhosus* are seen in the following table:



		30 Min.	3 Hrs.	6 Hrs.	24 Hrs.
Polynuclear cells	+ <i>B. typhosus</i> .....	∞	++++	+	0
" " inactivated	+ ".....	∞	∞	∞	∞
Supernatant fluid	+ ".....	∞	++	0	0
" " inactivated	+ ".....	++++	∞	∞	∞
Normal rabbit serum	+ ".....	++++	++++	∞	∞
" " inactivated	+ ".....	++++	∞	0	∞
N. r. s. + Polynuclear cells	+ ".....	++++	∞	∞	∞
Polynuclear cells.....		0	0	0	0
<i>B. typhosus</i> .....		++++	∞	∞	∞

∞ = Countless; + + + + = Very many; + + + = Many; + + + = Thousands; + + = Hundreds; + = Few; 0 = No colony

**Experiment 2.**—A 2,750-gm. rabbit was injected as before with casein 48 hours previous to bleeding. Polynuclear cells in the film stained by Giemsa's method, 90 per cent.

		30 Min.	3 Hrs.	6 Hrs.	24 Hrs.
Polynuclear cells	+ <i>B. typhosus</i> .....	+++++	+++++	∞	∞
" " inactivated	+ ".....	+++++	∞	∞	∞
Supernatant fluid	+ ".....	+++++	+++	++	+
" " inactivated	+ ".....	+++++	∞	∞	∞
Normal rabbit serum	+ ".....	++++	0	∞	0
" " inactivated	+ ".....	+++++	+++++	∞	∞
N. r. s. + Polynuclear cells	+ ".....	+++++	+++	++++	∞
Polynuclear cells.....		0	0	0	0
<i>B. typhosus</i> .....		∞	∞	∞	∞

**Experiment 3.**—A 1,800-gm. rabbit was injected as before with casein 32 hours previous to bleeding. Polynuclear cells in the film stained by Giemsa's method, 80 per cent.

		30 Min.	3 Hrs.	6 Hrs.	24 Hrs.
Polynuclear cells	+ <i>Sp. cholerae</i> .....	+++	++	0	0
" " inactivated	+ ".....	+++	+++	∞	∞
Supernatant fluid	+ ".....	+++	++	++	0
" " inactivated	+ ".....	+++++	∞	∞	∞
Normal rabbit serum	+ ".....	+++	++	0	0
" " inactivated	+ ".....	+++	+++++	∞	∞
N. r. s. + Polynuclear cells	+ ".....	+++	++	++++	∞
Polynuclear cells.....		0	0	0	0
<i>Sp. cholerae</i> .....		+++	+++++	∞	∞

The experiments on *B. typhosus* and *Sp. cholerae* show that polynuclear leukocytes have bactericidal power. This agrees with Petri's results (1904) as in his experiments with two rabbits the polynuclear cells killed *B. typhosus*.

**Experiment 4.**—A 1,800-gm. rabbit was injected as before with casein 48 hours previous to bleeding. Polynuclear cells in the film stained by Giemsa's method, 85 per cent.

		30 Min.	3 Hrs.	6 Hrs.	24 Hrs.
Polynuclear cells	+ <i>B. dysenteriae</i> .....	++++	++++	∞	∞
" " inactivated	+ ".....	++++	∞	∞	∞
Supernatant fluid	+ ".....	++++	++++	++	+
" " inactivated	+ ".....	++++	+++++	∞	∞
Normal rabbit serum	+ ".....	++++	++	0	0
" " inactivated	+ ".....	++++	+++++	∞	∞
N. r. s. + Polynuclear cells	+ ".....	+++	++++	++++	∞
Polynuclear cells.....		0	0	0	0
<i>B. dysenteriae</i> .....		++++	∞	∞	∞

**Experiment 5.**—A 1,800-gm. rabbit was injected as before with casein 18 hours previous to bleeding. Polynuclear cells counted, 80 per cent.

		30 Min.	3 Hrs.	6 Hrs.	24 Hrs.
Polynuclear cells	+ <i>B. dysenteriae</i> .....	+++++	∞	∞	∞
" " inactivated	+ " .....	+++++	∞	∞	∞
Supernatant fluid	+ " .....	+++++	+++++	+++	++
" " inactivated	+ " .....	+++++	+++++	∞	∞
Normal rabbit serum	+ " .....	+++++	+++++	++	0
" " inactivated	+ " .....	+++++	+++++	∞	∞
N. r. s. + Polynuclear cells	+ " .....	+++++	+++++	∞	∞
Polynuclear cells	.....	0	0	0	0
<i>B. dysenteriae</i> .....		+++++	∞	∞	∞

**Experiment 6.**—A 2,800-gm. rabbit was injected as before with casein 48 hours previous to bleeding. Polynuclear cells counted, 90 per cent.

		30 Min.	3 Hrs.	6 Hrs.	24 Hrs.
Polynuclear cells	+ <i>B. coli</i> .....	+++++	+++++	∞	∞
" " inactivated	+ " .....	+++++	∞	∞	∞
Supernatant fluid	+ " .....	+++++	+++++	+++	∞
" " inactivated	+ " .....	+++++	∞	∞	∞
Normal rabbit serum	.....	+++++	+++++	0	0
" " inactivated	+ " .....	+++++	∞	∞	∞
N. r. s. + Polynuclear cells	+ " .....	+++++	+++++	∞	∞
Polynuclear cells	.....	0	0	0	0
<i>B. coli</i> .....		+++++	∞	∞	∞

## II. MONONUCLEAR LEUKOCYTES.

**Experiment 7.**—A 2,800-gm. rabbit was injected as before with guinea-pig erythrocytes 32 hours previous to bleeding. Mononuclear cells counted, 50 per cent.

		30 Min.	3 Hrs.	6 Hrs.	24 Hrs.
Mononuclear cells	+ <i>B. typhosus</i> .....	+++++	∞	∞	∞
" " inactivated	+ " .....	+++++	∞	∞	∞
Supernatant fluid	+ " .....	+++++	+++++	+++	0
" " inactivated	+ " .....	+++++	∞	∞	∞
Normal rabbit serum	+ " .....	+++++	+++++	++	0
" " inactivated	+ " .....	+++++	∞	∞	∞
N. r. s. + Mononuclear cells	+ " .....	+++++	∞	∞	∞
Mononuclear cells	.....	0	0	0	0
<i>B. typhosus</i> .....		+++++	∞	∞	∞

**Experiment 8.**—A 2,800-gm. rabbit was injected as before with guinea-pig erythrocytes 48 hours previous to bleeding. Mononuclear leucocytes counted, 40 per cent.

		30 Min.	3 Hrs.	6 Hrs.	24 Hrs.
Mononuclear cells	+ <i>Sp. cholerae</i> .....	+++++	+++++	∞	∞
" " inactivated	+ " .....	+++++	∞	∞	∞
Supernatant fluid	+ " .....	+++++	+++++	+++	++
" " inactivated	+ " .....	+++++	∞	∞	∞
Normal rabbit serum	+ " .....	+++++	++	+	0
" " inactivated	+ " .....	+++++	+++++	∞	∞
N. r. s. + Mononuclear cells	+ " .....	+++++	+++++	∞	∞
Mononuclear cells	.....	0	0	0	0
<i>Sp. cholerae</i> .....		+++++	∞	∞	∞

**Experiment 9.**—A 2,300-gm. rabbit was injected as before with guinea-pig erythrocytes 24 hours previous to bleeding. Mononuclear cells counted, 50 per cent.

		30 Min.	3 Hrs.	6 Hrs.	24 Hrs.
Mononuclear cells	+ <i>B. dysenteriae</i> .....	+++++	∞	∞	∞
" " inactivated	+ ".....	+++++	∞	∞	∞
Supernatant fluid	+ ".....	+++++	+++	+++	0
" " inactivated	+ ".....	+++++	+++++	∞	∞
Normal rabbit serum	+ ".....	+++++	+++	+	0
" " inactivated	+ ".....	+++++	+++++	∞	∞
N. r. s. + Mononuclear cells	+ ".....	+++++	+++++	∞	∞
Mononuclear cells	.....	0	0	0	0
<i>B. dysenteriae</i> .....		+++++	∞	∞	∞

**Experiment 10.**—A 1,950-gm. rabbit was injected as before with guinea-pig erythrocytes 24 hours previous to bleeding. Mononuclear cells counted, 45 per cent.

		30 Min.	3 Hrs.	6 Hrs.	24 Hrs.
Mononuclear cells	+ <i>B. coli</i> .....	+++++	+++++	∞	∞
" " inactivated	+ ".....	+++++	∞	∞	∞
Supernatant fluid	+ ".....	+++++	+++++	+++	++
" " inactivated	+ ".....	+++++	∞	∞	∞
Normal rabbit serum	+ ".....	+++++	+++	++	0
" " inactivated	+ ".....	+++++	+++++	∞	∞
N. r. s. + Mononuclear cells	+ ".....	+++	+++	+++++	∞
Mononuclear cells	.....	0	0	0	0
<i>B. coli</i> .....		+++++	∞	∞	∞

### III. ORGAN EXTRACTS.

		30 Min.	3 Hrs.	6 Hrs.	24 Hrs.
Spleen extract	+ <i>B. typhosus</i> .....	+++++	∞	∞	∞
Liver " + "	.....	+++++	+++++	∞	∞
Marrow " + "	.....	+++++	+++++	∞	∞
Spleen " + <i>Sp. cholerae</i>	.....	+++	+++++	∞	∞
Liver " + "	.....	+++++	+++++	∞	∞
Marrow " + "	.....	+++	∞	∞	∞
Spleen " + <i>B. dysenteriae</i>	.....	+++++	∞	∞	∞
Liver " + "	.....	+++++	∞	∞	∞
Marrow " + "	.....	+++	+++++	∞	∞
Spleen " + <i>B. coli</i>	.....	+++++	+++++	∞	∞
Liver " + "	.....	+++++	∞	∞	∞
Marrow " + "	.....	+++++	∞	∞	∞

### CONCLUSIONS.

The following general conclusions may be drawn from the experiments I have described:

1. No bactericidal substance for *B. typhosus*, *Sp. cholerae*, *B. dysenteriae*, and *B. coli* ordinarily exists in the polynuclear leukocytes obtained from the normal adult rabbit; however, one experiment on *B. typhosus* and one on *Sp. cholerae* showed some bactericidal effect for them.

2. No bactericidal substance for *B. typhosus*, *Sp. cholerae*, *B.*

*dysenteriae*, and *B. coli* exists in the mononuclear leukocytes obtained from the normal adult rabbit.

3. No bactericidal substance for *B. typhosus*, *Sp. cholerae*, *B. dysenteriae*, and *B. coli* exists in the intracellular products of the spleen, liver, and bone-marrow of the normal rabbit.

4. Normal rabbit serum and supernatant fluid are invariably powerful in their bactericidal effect on *B. typhosus*, *Sp. cholerae*, and *B. dysenteriae*, but when heated at 55° C. to 60° C. for 30 min. the serum fails in bactericidal action.

As previously, I have applied a disintegrating method to get bactericidal substance in the body of leukocytes, but the result did not show distinctly any substance similar to "alexin" or "complement;" therefore I question whether the leukocytes have an endolysin as the bactericidal substance. Concerning this I also have doubt as to the bactericidal substance in the leukocytes.

The pleasant duty is incumbent upon me, in conclusion, of recording my sense of indebtedness to Prof. S. Kitasato for his kind suggestion; and I must also thank Prof. K. Shiga for similar services.

## THE TUBERCLE BACILLUS IN MILK.\*

GUTHRIE McCONNELL.

(From the Bacteriological Laboratory, State Board of Health of Missouri, St. Louis, Mo.)

ALTHOUGH the question as to the degree of danger of infection by bovine tuberculosis is a mooted one, nevertheless there can be no hesitation in acknowledging that the purer the milk supply the better protected is the health of the community. Milk that contains bovine tubercle bacilli is not as wholesome a food as that which is free from them, but if they are to be present it is better that they should be dead than living.

About a year ago a few investigations were made concerning the milk supply of the city of St. Louis and the results that were obtained were both interesting and in a way satisfactory.

According to the experiments of Rosenau<sup>1</sup> it was shown that the tubercle bacillus in milk loses its infective properties for guinea-pigs when heated to 60° C. and maintained at that temperature for 20 minutes, or to 65° C. for a much shorter time. To quote further, it should be remembered that the milk in these tests was very heavily infected with virulent cultures, as indicated by the prompt death of the control animals. Under natural conditions milk would practically never contain such an enormous amount of infection. It is justifiable to assume that if 60° C. for 20 minutes is sufficient to destroy the infectiveness of such milk when injected into the peritoneal cavity of a guinea-pig, any ordinary market milk after such treatment would be quite safe for human use by the mouth, as far as tubercle bacilli are concerned.

In commercial practice the above results are sought for in a different way. Prolonged heating of milk even at comparatively low temperatures produces certain changes that may interfere to some extent with its value as a food. Another consideration is that of the practical impossibility of uniformly heating the large quantities of milk that are handled by the larger dealers. The result is that the larger companies make use of that form of pasteurization known as the "flash" method. Various forms of apparatus have been devised for this

\* Received for publication February 26, 1909.

<sup>1</sup> *Bull. No. 42, Hyg. Lab., U. S. Pub. Health and Mar. Hosp. Ser., 1908*



purpose but the principle in most of them is more or less the same. The milk is allowed to pass upon a rapidly turning plate, the centrifugal force spreading the milk in an extremely thin layer, less than  $\frac{1}{100}$  of an inch at the periphery. Under this disc is a hot-water chamber kept at the proper temperature by means of steam jets. The extreme thinness of the film of milk permits it to be heated to a high point almost instantaneously. The milk then passes over plates cooled by brine and goes on to the receiving tank, the entire process taking from 30 seconds to one minute. The degree of heat employed is in the neighborhood of from 70° to 80° C., or slightly lower if the exposure is to be longer.

All of the larger dealers make it a common practice to pasteurize their milk during the winter as well as the summer months so that there is no time during the year when the milk is not so treated.

On February 1, 1908, the following experiments were made, the idea being that of testing some milk as it was brought into the city and then other samples of the milk as they were distributed. The first sample was obtained from the can just after the milk train arrived in the city. This specimen came from a lot of three cans that contained the milk from one small farm. The second, obtained at the same time, was from a lot of some 30 cans that came from a general station where milk from numerous sources was received in bulk. A third specimen was taken at a dairy from a tank holding some 800 gallons, about one-half of which had been once pasteurized but had been mixed with nearly an equal amount of raw milk. The material in this tank represented milk that had come from many different farms and consequently hundreds of different cows. The fourth specimen was the same as No. 3 after it had passed through the commercial flash pasteurizer.

About a quart of milk was taken as a sample, this being centrifuged as soon as it reached the laboratory. Those samples from the train cans contained quite a large amount of sediment but those from the dairy had but little. This sediment was diluted with the respective milk and then portions were injected, subcutaneously, into the abdominal wall of each of four guinea-pigs in each experiment. Each set of guinea-pigs was kept in its separate cage and all under the same environment. The following tables show the results.

TABLE 1.  
RAW MILK FROM ONE SOURCE.

	2.1.'08	2.8.'08	2.21.'08	3.4.'08	3.10.'08	Autopsy
A.	270 gm.	240 gm.	Died 2.18.'08.			Negative
B.	280 "	250 "	335 gm.	350 gm.	380 gm.	"
C.	260 "	230 "	310 "	320 "	390 "	"
D.	390 "	370 "	480 "	510 "	540 "	"

In this series Pig A lost weight and died in 17 days but no macroscopic lesions were found. The other pigs showed no changes when chloroformed some six weeks later.

TABLE 2.  
RAW MILK FROM SEVERAL SOURCES.

	2.1.'08	2.8.'08	2.21.'08	3.4.'08	3.10.'08	Autopsy
A.	360 gm.	365 gm.	430 gm.	470 gm.	460 gm.	Enlarged nodes
B.	290 "	280 "	310 "	360 "	380 "	Negative
C.	310 "	350 "	410 "	460 "	490 "	"
D.	280 "	Died 2.4.'08.				

In this series pig A, which was chloroformed about six weeks after the injection, showed several enlarged lymph nodes under the jaw in the region of the trachea. On the left side there was a node about 2 cm. in diameter; other adjacent nodes were enlarged but not to the same extent. No lesions were found in the abdominal or pleural cavities.

Pig D died three days after the inoculation.

TABLE 3.  
ONE-HALF RAW, ONE-HALF PASTEURIZED MILK.

	2.1.'08	2.8.'08	2.21.'08	3.4.'08	3.10.'08	Autopsy
A.	310 gm.	290 gm.	350 gm.	395 gm.	430 gm.	Negative
B.	400 "	390 "	420 "	470 "	500 "	Enlarged nodes under chin
C.	370 "	400 "	460 "	480 "	525 "	" " " "
D.	320 "	360 "	360 "	390 "	440 "	" " " "

Pig A. showed nothing abnormal by the end of six weeks.

Pig B (3.14.'08) on the right side of the neck under the chin presented an enlarged node about 2 cm. long and 1 cm. in diameter. Several other nodes, smaller than the above, but nevertheless enlarged, were found. Two somewhat enlarged bronchial nodes were found on the right side. Pleural and peritoneal cavities negative.

Pig C (3.13.'08): Tracheal nodes enlarged to size of cherries. At the site of the injection into the abdominal wall there was a swelling about 0.5 cm. in diameter free from hair and filled with a thick caseous material. Bronchial nodes were enlarged. In the right lung there was a small yellowish area. No lesions in the abdominal cavity.

Pig D (3.14.'08): On the left side under the chin there was present an enlarged node about 1 cm. long by 0.5 cm. thick. On the right there was a somewhat smaller node. No lesions were found in either the pleural or peritoneal cavities.

TABLE 4.  
PASTEURIZED MILK.

	2.1.'08	2.8.'08	2.21.'08	3.4.'08	3.10.'08	Autopsy
A.	340 gm.	340 gm.	370 gm.	375 gm.	400 gm.	Negative
B.	225 "	250 "	280 "	300 "	340 "	"
C.	280 "	290 "	300 "	285 "	320 "	"
D.	260 "	270 "	280 "	280 "	310 "	"

Pig A was chloroformed some six weeks after the inoculation (on 3.13.'08).

Pigs B, C, and D four days later than the above. In no instance was anything abnormal found at the site of the inoculation, in the pleural or in the peritoneal cavities.

Whenever enlarged nodes were present they were found to consist of a rather dry caseous mass contained within a thin capsule apparently composed of connective tissue and a narrow zone of lymphoid tissue.

The results in these series seem to follow a fairly logical course. In Series 1 where the milk was obtained from a single source none of the pigs showed any lesions. This could easily be explained on the basis that that particular herd was free from tuberculosis.

In Series 2 the raw milk came from several sources, in this way permitting the larger mass of milk to become contaminated by infected material from some one source. In this we find one of the three guinea-pigs involved. The fourth pig, dying within three days after the inoculation, has to be omitted.

Series 3 shows what naturally might be expected, when the number of sources from which so large a quantity of milk is obtained is taken into consideration. In this instance a comparatively small amount of infected matter would be able to pollute the larger mass. In this series three out of the four pigs showed marked involvement of the lymph nodes of the neck.

In Series 4 where pasteurized milk was employed none of the four pigs showed lesions.

Although the number of experiments is small yet it would seem to bring out two points of interest. The first, which is very evident, is that the greater the number of sources from which the milk is derived the greater the likelihood of infection by tuberculosis. The second, that commercial pasteurization by the flash method, when properly carried out, is sufficient to destroy the organism of tuberculosis.

The question as to the thermal death-point of the tubercle bacillus is thoroughly discussed in the article by Rosenau. The early investigators thought that the tubercle bacillus was a spore-producer and was able to withstand very high degrees of heat. As methods became more exact and observers more accurate in their conclusions it was found that the tubercle bacillus could be destroyed by comparatively low temperatures after short exposures. Six observers besides Rosenau report the death of tubercle bacilli after an exposure to 60° C. for 20 minutes. Russell and Hastings<sup>1</sup> showed that material exposed in a continuous-action pasteurizing machine to 71° C. for one minute was freed from living tubercle bacilli.

<sup>1</sup> 21st Ann. Rep., Univ. of Wisconsin Agri. Exper. Sta., 1904.

# CULTURES OF TUBERCLE BACILLI ISOLATED FROM MILK.\*<sup>1</sup>

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IN an examination of the milk supply of New York City made by me over a year ago, 17 samples among 107 of milk retailed from 40-quart cans were found to contain tubercle bacilli. A full report of this study, together with a consideration of the welfare of the children who drank this contaminated milk, has been published elsewhere.<sup>2</sup> In the course of this work it was considered that it might be of interest to isolate some of these strains of tubercle bacilli, and to determine their cultural and pathogenic characteristics. It was to be expected, considering the source of the material, that probably all the strains would prove to be bovine in type. However, there seemed a possibility, owing to the great degree of exposure to which such milk is subject in its journey from the cow to the retailer, that contamination with bacilli from human source might occur. In the hope of encountering such a case cultures were undertaken. It seemed also of interest to inquire whether the cultures would conform strictly to definite types or whether intermediate varieties, such as have been met with by some others, would be isolated.

The technique employed was, in brief, as follows:

Ten c.c. of milk was centrifugalized, and 1 c.c. of the cream injected into a guinea-pig; 1 c.c. of the lowest skimmed milk was inoculated into another guinea-pig. In some cases one of these pigs developed tuberculosis, in other instances both of them. Of the cultures which I now report, five were made from pigs inoculated with cream, three from animals inoculated with sediment. I shall not detail the method of obtaining cultures, as it is the one generally in use, and similar to that employed by me in previous studies.<sup>3</sup>

In some cases coagulated dog serum was used as a medium, as first suggested by Theobald Smith, but in most instances the tissue was transferred directly to Dorset's egg media. In two instances, not included in this series, culture was attempted but failed; in two others the growth was very scanty, and after the third generation the strain was lost.

\* Received for publication April 10, 1909.

<sup>1</sup> Read before the New York Pathological Society, January 13, 1909.

<sup>2</sup> *Jour. Amer. Med. Assn.*, 1909, 51, p. 1011.

<sup>3</sup> *Archiv. Pediat.*, 1908, p. 31; *Amer. Jour. Med. Sci.*, 1908, 136, p. 1831.

The results are given in the appended table. A review of this summary shows that seven cultures induced a fatal generalized tuberculosis in the rabbit, whereas one possessed but slight virulence for this species. For these tests intravenous inoculations of standard emulsions were employed, excepting in the last case, where it will be noted that 1 mg. of culture was inoculated. This strain was isolated and studied by Dr. Woglam and Dr. Krumweide, whom I take pleasure in thanking for this work.

The cultures I-VII must be classed as bovine in type, not only on account of the marked virulence to rabbits which they exhibited, but also because of their cultural characteristics. They grew very sparsely, showing for some generations only a fine veil-like growth, which it was very difficult or impossible to transfer successfully to glycerin broth. They were markedly similar in all these properties, only one standing apart from the others in that it showed a more abundant growth in the first generation. However, even this strain did not grow profusely when compared with cultures of the human variety.

Culture VIII is of especial interest and seems worthy of detailed description. The original material was obtained from a can of milk in a small grocery store. It was inoculated subcutaneously into two guinea-pigs, both of which developed generalized tuberculosis. From one of these animals tissue was inoculated into another pig (No. 855), from which cultures were made upon egg media about two months later. In order not to lose the material, tissue was once more transferred into a pig (No. 972), and further cultures carried out after the same interval. These two sets of cultures proved to be identical; after three weeks a vigorous confluent growth was obtained on glycerin egg. These results were so unexpected that a rabbit (No. 625) was inoculated with one of these strains, and cultures were made from its tissues. By using the rabbit instead of the highly susceptible guinea-pig we made certain of selecting for culture the most virulent bacilli. These strains also grew vigorously in a manner characteristic of bacilli of the human type. Reference to the virulence tests of the table shows that this strain (Culture VIII) in strong contrast to all the others, possessed but feeble virulence for rabbits. One of the rabbits when inoculated weighed 1,740 gm.,



and when killed had gained 550 gm., the other weighed 1,710 gm., and now, after a period of 63 days, weighs 1,820 gm. Thus this culture, in contradistinction to the others, must be regarded as belonging to the human type, both on account of its facility for cultivation and its comparatively feeble virulence.

From a study of these eight cultures we must emphasize the sharp contrast which usually exists between tubercle bacilli of the human and those of the bovine type, isolated from cattle, a distinction which in the instances here reported was absolutely diagnostic and incapable of misinterpretation. Furthermore the results are instructive from another point of view, for, for the first time, they bring forth an instance where tubercle bacilli of the human variety have been isolated from milk and thus point out another source of danger from contamination by the tuberculous individual.

TABLE 1.  
DATA OF INTRAVENOUS INOCULATION OF RABBITS WITH PURE CULTURES OF TUBERCLE BACILLI.

	Total Age of Culture	Genera- tion	Age of Culture	Amount Inocu- lated	No. Rabbit	Result	General Remarks
Culture I ..	82 days	3d	22 days	0.5 c.c.	320	Chloroformed 21 days (ill)	General tuberculosis
" II ..	95 "	4th	20 "	0.5 c.c.	321	Died 18 days	" "
" III ..	76 "	3d	25 "	0.5 c.c.	325	" 16 "	" "
" IV ..	67 "	3d	28 "	0.4 c.c.	326	" 20 "	" "
" V ..	98 "	4th	21 "	0.5 c.c.	327	" 23 "	" "
" VI ..	79 "	3d	30 "	0.5 c.c.	331	Chloroformed 17 days (ill)	" "
" VII ..	69 "	3d	26 "	0.5 c.c.	332	Died 15 days	" "
" VIII }	21 "	3d	63 "	1 mg.	181	Killed 80 days	Few tubercles in lungs and kidneys
	21 "	2d	42 "	1 mg.	521	Alive 63 days	Gained in weight

## SOME CHARACTERISTICS OF THE STREPTOCOCCI FOUND IN SCARLET FEVER.\*†

BERTHA VAN H. ANTHONY.

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THE following work was undertaken to determine the frequency of the occurrence of the hemolysing streptococcus in the blood, pus, blister fluid, and throats of scarlet fever patients, its relative proportion to other streptococci present, and the persistence of its hemolysing power. By the term "hemolysing streptococcus" is meant that spherical or spheroidal organism which, by dividing in only one plane, grows in long or short chains, the individuals usually occurring in pairs, and which gives a more or less definite area of hemolysis about each colony when plated out on blood agar.

In 1903 Schottmüller,<sup>1</sup> and soon after, Rosenow,<sup>2</sup> showed that pneumococcus colonies gave green color in blood agar poured plates, *Strept. pyogenes* gave hemolysing colonies, and other streptococci (*Strept. viridans*), green colonies. Ruediger,<sup>3</sup> working with various throat cultures, found that pneumococci showed green colonies and sometimes hemolysing colonies; *Strept. pyogenes*, hemolysing colonies; and other streptococci, green colonies. He also found that hemolysing colonies may show green color in subsequent plates, and that slightly hemolysing colonies and those with greenish cast, while very puzzling, should be distinguished from *Strept. pyogenes*. Moreover, he found but a small proportion of the green colonies occurring in scarlet fever and other throat cultures to be pneumococcus. The others seemed to belong to a large group lying between *Strept. pyogenes* and the pneumococcus, some being very like *Strept. viridans* of Schottmüller and others being related to pneumococci.

Ruediger explains the green coloration in blood plates as due to the formation of lactic acid, which acts then on the sugars in the blood medium. In glucose-agar blood plates *Strept. pyogenes* does give green colonies entirely, but none in blood and plain agar because it

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† This work was carried out under the direction of Drs. W. H. Park and A. W. Williams.

Müsch. med. Wchnschr., 1903, 50, pp. 849, 909.

<sup>3</sup> Ibid., 1906, 3, p. 663.

<sup>2</sup> Jour. Infect. Dis., 1904, 1, p. 308.

does not ferment muscle sugar readily and the hemolysis outruns the green coloration. In our laboratory all the hemolysing strains, and also the green ones, when tested and retested in glucose-agar blood plates, gave green colonies.

This plate method was the chief means of isolating the streptococci in pure culture from the scarlet fever material. In the case of blood about 5 or 6 c.c. were drawn from the veins of the arms of living patients, and in autopsy cases from the heart by means of a sterile syringe.

The blister fluid was obtained by applying strong ammonia to a small area of the skin where the rash was brightest, usually on the chest, by means of a disk of blotting paper surrounded by a circle of vaseline and held firmly in place by crossed adhesive strips arranged in a star form, so that the air was practically excluded. After 10 or 15 minutes the fluid in the blister thus formed was drawn with sterile capillary pipettes which penetrated the delicate epidermis easily. Cultures were made in serum broth, and, after 24 hours' incubation, were plated out in plates made with horses' blood.

The pus was drawn by a sterile syringe from suppurating glands or other foci of infection. Dilutions were made, but were plated out at once because the number of organisms present was usually much greater than in the blood.

Throat cultures were taken by means of sterile cotton swabs rubbed thoroughly on both tonsils. The swab of each case was beaten in a tube of plain broth and dilutions made before plating.

The following table gives in condensed form the number of cases examined and strains isolated in pure culture, from material taken from early scarlet fever and measles cases.

TABLE 1.  
STREPTOCOCCI IN SCARLET FEVER.

SOURCE	NUMBER OF CASES TESTED	NO GROWTH	STREPTOCOCCI ISOLATED	BLOOD PLATES	
				<i>Strept. pyogenes</i> ? Hemolysing	Other Streptococci
Autopsy blood (from heart) . . .	18	8	10	+	—
Living blood (from arm) . . . . .	16	15	1	—	+
Total . . . . .	34	..	11		
Autopsy pus . . . . .	10	3	7	+	—
Living pus . . . . .	9	0	9	+	+
Total . . . . .	19	..	16		1 colony in 1 case
Blister fluid . . . . .	26	23	3	+	+
Scarlet throats . . . . .	35 (5 rejected)	0	30	+	+
				(22 cases)	(26 cases)
CONTROLS:					
Measles throats . . . . .	24	..	24	+	+
				(20 cases)	(24 cases)
Diphtheria throats . . . . .	4	..	4	+	+
				(a few colonies in one case)	(4 cases)

The percentage of hemolysing colonies in the different strains of both scarlet and measles throats varied markedly, namely, from zero to one hundred. Of the 30 scarlet fever throats, 8 cases showed no hemolysing colonies at all; 4 showed no green colonies, and 18 showed both green and hemolysing colonies. Of the 24 measles throats, all showed green colonies and 4 showed no hemolysing colonies. There was so much variation in color reaction and the area of hemolysis, that the following terms were used in an attempt to classify the colonies:

Hemolysing, small hemolysing, very small hemolysing.

Slightly hemolysing (large and small).

Doubtful.

Green (light and dark).

Colonies were fished and replated during successive generations and it was found that they varied up and down the scale.

Hemolysing colonies varied in area of hemolysis.

Green colonies varied in shade of green.

Small hemolysing colonies became large or slightly hemolysing, or green; this green in subsequent generations became hemolysing again.

Slightly hemolysing colonies remained the same, became doubtful, or showed green in later plates.

Doubtful colonies became slightly hemolysing, remained doubtful, or showed green.

The 30 streptococcus strains isolated from blood, pus, and blister fluid of scarlet fever cases were studied with two controls: C. 100, a Bellevue Laboratory culture, and C. 101, from a case of cellulitis after erysipelas. In contrast to the throat cultures, these strains from blood and pus, when tested in pure culture in blood plates, each gave good hemolysing colonies with one exception.<sup>1</sup> The exception was the only strain isolated from living blood, and it gave green colonies alone. The throat culture in this case also showed green colonies only. The patient from whom these cultures were derived had a good rash, ran a typical course of scarlet fever, and recovered without complications.

The three blister fluids which yielded streptococci showed both hemolysing and green colonies. These children recovered also.

In eight of the autopsy cases where both blood and pus were taken from the one subject, both materials were positive in four. In the

<sup>1</sup> In addition, the pus from the cervical gland in one living case, Sc. 95, showed one green colony which remained green when retested twice.

other four the blood was negative in three with the pus positive in two. One case had negative pus and positive blood; another showed both the blood and pus negative. Blister fluid was obtained in only two cases from which blood and pus were taken later at autopsy. In both these cases the blister fluid was negative.

#### RETEST OF THE HEMOLYSING POWER OF STREPTOCOCCUS STRAINS.

Twenty-five of these strains from scarlet fever cases and the two controls, C. 100 and C. 101, were kept in the ice-box on blood agar slants and transferred at about four-week intervals, for three months to a year. They were then retested in successive blood plates and showed marked variation as to hemolysis though all, except 51 Blood and Throat (which were green when isolated and remained so), had been fished from colonies which showed good hemolysis in the original. The retest was carried on as follows:

**Technic.**—From a 24-hour blood agar slant of each strain a very small portion of the growth was removed with a straight platinum needle and put in a 5 c.c. broth tube. This was shaken thoroughly and the two loopfuls of the broth transferred at once to a tube containing 1 c.c. of sterile horse blood. Melted agar was added and the plates poured. These plates were incubated 18 to 24 hours and the colonies showing least amount of hemolysis were fished on blood agar slants which were plated out in turn. A careful record was kept as to the size and reaction of the colonies of each strain, only the most variable fishings being carried on from one plating to another. Each broth tube, from which an agar plate was made, was incubated and a smear from it examined, so no contamination could creep in unawares.

From the hemolysing colonies arose:

1. Hemolysing colonies of similar size.
2. Slightly hemolysing (large and small).
3. Doubtful colonies.
4. Green (light and dark).

In subsequent platings, as in the case of fishings from the original throat cultures, there was a marked variation up and down the scale. Hemolysing colonies varied in hemolysis, became slightly hemolysing, doubtful, green, or showed no green nor hemolysis. Slightly hemolysing colonies became hemolysing, remained slightly hemolysing, or were doubtful, green, or showed no green nor hemolysis.

Green colonies remained green for the most part, but a few returned to hemolysing, slightly hemolysing colonies, or were doubtful.

In two strains, 53 Blood and Sc. 48 Blood, at the fourth and third platings, respectively, there occurred among other colonies some



which showed neither hemolysis nor green color. These were carefully fished and found in smears of broth transfers to be pure streptococcus. When these fishings were replated and refished one or more times they gave rise to hemolysing, slightly hemolysing, green, and doubtful, sometimes all in the same plate. Only a few remained the same, that is, showed no green nor hemolysis.

Eight fishings from these colonies (of 53 Blood), which showed no green color and no hemolysis, were tested simultaneously in blood plates of plain agar, peptone water agar, and sugar-free agar (made from broth with colon bacillus added and resterilized), with the result that in the plain and peptone agar the hemolysis returned for the most part, while in the sugar-free agar the colonies remained the same or showed but slight trace of hemolysis.

These colonies (showing no green nor hemolysis) were tested also upon: Glucose agar which gave green colonies; glycerin agar which gave hemolysing and slightly hemolysing colonies; beerwort agar which gave deep-green colonies.

Two strains, 13 Blood and Pus, were kept on glucose-agar blood slants for a number of transfers at several-day intervals and when changed suddenly to plain agar blood plates showed no loss of hemolysing power.

The two control cultures, one (C. 100) from the laboratory of Bellevue Hospital, and the other (C. 101) isolated recently from a case of cellulitis following erysipelas, showed but little variation, for only two slightly hemolysing colonies occurred in the six platings of C. 100 and none in C. 101.

A retest was made also of 20 fishings from scarlet fever throat cultures. Control Sc. 40 was from throat of an erysipelas case which developed a rash and was suspected of being scarlet fever, but later proved negative. These strains had been kept in the ice-box on blood agar slants for 12 to 18 months, and comprised not only hemolysing, but hemolysing and green colonies when first isolated. The reactions of the originally hemolysing colonies followed practically those showed of the blood and pus hemolysing colonies when they were retested. The colonies which had been green when isolated remained so in every replating except once in the case Sc. 87, where doubtful colonies occurred in the third plate, but when these were fished and plated

again they showed green and remained so for subsequent platings.

In the replating of the two slightly hemolysing strains, Sc. 81, throat, and Sc. 88, throat, greater variation occurred. *Sc. 88 slightly hemolysing* (large) gave: Hemolysing, slightly hemolysing, and a few doubtful colonies, which, however, became hemolysing again. On the other hand, *Sc. 81 slightly hemolysing* gave but a low percentage of hemolysing colonies and these were small in size. The majority of colonies were slightly hemolysing with a few doubtful and a number of green ones. These last, the green, became small and very small hemolysing colonies four times, but for the most part gave rise to slightly hemolysing and still other green colonies.

The control from the erysipelas throat, which was hemolysing when isolated, gave, in the retest, large hemolysing colonies.

#### SUMMARY.

Although the power of hemolysing streptococci to cause hemolysis seems to be a slightly variable quality and is certainly dependent upon conditions we do not understand, yet there is a distinct tendency in this power to continue, even though the strains be kept on artificial media, for in the retest the majority of colonies were hemolysing, the variation from the original strain being usually about 5 per cent, or less, in the first replating. In these and subsequent plates only the most variable colonies were fished and even then the percentage of hemolysing colonies was usually greater than 25 per cent, sometimes running between 75 and 100 per cent. All strains were plated out at least six successive times and some, eight or nine times. Any variation occurring cannot be due entirely to long cultivation on artificial media, partly because some small hemolysing colonies from throat cultures changed rapidly to green after isolation; and also some recent green colonies, derived from old hemolysing cultures kept on agar for seven to twelve months, have regained their hemolysis once more. To be sure, when some of these apparently accidental green colonies, which formed but a very small part of all the colonies present, were selected strains were brought out which had lost their hemolysis completely. This occurred in  $33\frac{1}{2}$  per cent of the 25 Blood and Pus cultures retested, and these green strains failed to regain any appear-

ance of hemolysis, though they were plated out six successive times. It is doubtful if these can longer be classed with *Strept. pyogenes*. On the other hand, as cited above in Sc. 48 Blood, some green colonies show a quick change to hemolysis again.

The occurrence of hemolysing streptococci (*Strept. pyogenes*?), as seen in Table 1, was almost constant in the material taken from scarlet fever, measles, and diphtheria throats, while *Strept. viridans* was always present.

In scarlet fever cases the hemolysing streptococcus alone<sup>1</sup> occurred in the pus from cervical glands and other suppurating foci in all of the nine living cases, and was recovered in 7 of the 10 autopsies.

In the living blood and blister fluid streptococci would seem to be rather infrequent since they were found in but 3 out of 26 blisters and once in 16 samples of living blood. It is interesting to note that the three children from whom streptococci, both hemolysing and viridans, were isolated by blistering and also the man whose blood contained *Strept. viridans*, all recovered.

Streptococci were not found in all cases in which blood was examined after death, being present in but 10 of the 18 cases tested. These were of the hemolysing type.

<sup>1</sup> One case had one green colony, which remained green when retested.

# AN EPIDEMIC AMONG ENGLISH SPARROWS DUE TO *BACILLUS CLOACAE*.\*

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THE frequent outbreaks of sickness among farm poultry, together with the occasional epidemics which occur in the aviaries of bird-fanciers, have led to a more or less careful study of the cause of these diseases.

In 1893 Klein<sup>1</sup> examined a number of pheasants which succumbed during the rearing at Hawarden, England, and isolated a bacillus which he describes as resembling very closely *B. coli*. The only differences noted between the organism found in the pheasants and *B. coli* were that the former was more actively motile, shorter, and did not coagulate milk.<sup>2</sup> Two young pheasants, four young chickens, two pigeons, two half-grown chickens, two rabbits, and two guinea-pigs were inoculated with pure cultures. The two pheasants were found dead the next day. All the other animals survived. Sanfelice<sup>3</sup> obtained an organism from infected pigeons which he thought identical with *B. coli*. The bacillus was obtained from the liver, spleen, and heart's blood. Pigeons inoculated subcutaneously with 1 to  $\frac{1}{2}$  c.c. of a broth culture of this organism developed abscesses at the point of inoculation, but pigeons, inoculated intraperitoneally with even smaller doses, died in 24 hours. Kern<sup>4</sup> studied an epidemic which occurred among canaries in Budapest. Two new birds were bought and introduced into the aviary of the veterinary academy and in 22 days all but three of the birds in the aviary had died one after the other. Birds were found on the floor of the cages doubled up or on their backs. A bacillus was isolated in pure culture from the heart's blood, and other birds were inoculated. Some of these died in from four to six days. Pigeons and chickens were inoculated with negative results. Some positive and some negative results were obtained with sparrows. The specific bacteria were recovered from birds which died from the injection experiments. Tartakowsky described an epidemic which occurred among the small birds of St. Petersburg and isolated an organism to which he gave the name of "*Bacillus laxiacidae*." He called the disease seticemic enteritis of sparrows. The bacillus was said to resemble *B. coli* in its characteristics; but it had some resemblance also to *B. psittacosis* so that this author was undecided as to which class it belonged. Miessner<sup>5</sup> and Schern, in November, 1906, investigated the cause of death of a number of canaries and isolated, from the spleen and liver of these birds, an organism which they consider to be different from any previously described. The organism is described as a plump, non-motile bacillus rounded on the ends. It stains fairly well with ordinary stains but is negative to Gram. It grows on all culture media, but better on glycerin agar or in glycerin broth. These

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<sup>1</sup> *Jour. Path. and Bact.*, 1894, 11, p. 214.

<sup>2</sup> Klein does not state the time for which the milk was incubated.

<sup>3</sup> *Ztschr. f. Hyg.*, 1895, 20, p. 23.

<sup>5</sup> *Centralbl. f. Bakt.*, 1899, 25, p. 80

<sup>4</sup> *Deut. Ztschr. f. Tiermed.*, 1896, 22, p. 171.

<sup>6</sup> *Arch. f. wissenschaft. u. prakt. Thierh.*, 1908, 34, p. 133.

authors state that the organism was obtained in every case from the liver and spleen, but never from the blood of the birds. They decided that the organism so discovered was a "Bacterium sui generis," and burdened it with the name *B. canariensis necrophorus*. Zwick<sup>1</sup> studied the cause of death of a number of canaries which succumbed during an epidemic which occurred in the cages of one of the bird-fanciers of Stuttgart, Germany. He isolated an organism from the blood of the birds which he described as a small, non-motile bacillus, rounded on the ends and negative to Gram. Birds which were inoculated with this organism are said to have died in from 24 to 48 hours after the injection. Rettger and Harvey<sup>2</sup> have recently isolated an organism from young chickens which had died from so-called "white diarrhea." The organism is described as a long, slender, non-motile bacillus, slightly rounded on the ends, which, in its microscopic appearance, resembles *B. typhosus*. On agar slants, according to these authors, growth is visible in 24 hours, but it spreads little and remains delicate even after prolonged incubation. On potato, an almost invisible streak is produced along the whole line of inoculation. In litmus milk, little or no change occurs within the first 48 hours. Thereafter, the milk becomes slightly acid; but it is not coagulated. Gelatin is not liquefied. Gas is produced in dextrose fermentation tubes, but maltose, lactose, saccharose, inulin, and dextrin, according to these authors, are not fermented.

The bacterial infection of birds, considered in this paper, was observed in 23 English sparrows, *Passer domesticus*, which were caught in a figure-four trap and transferred to large cages without any unnecessary handling. All the birds, which were captured for another purpose, were apparently healthy when placed in the cages. The cages were carefully cleaned and disinfected before the birds were placed in them and again after the death of any of the birds, before any more were introduced. Every care was taken of the birds, but they continued to die, in from four days to two weeks after being brought into captivity.

The symptoms in all cases were similar. When first put into the cage, the birds were extremely active. In two or three days, they became quiet, lost their appetite, stood on the floor with feathers ruffled up and eyes closed. They became extremely weak. Toward the end, if placed on their backs, they were too weak to right themselves. In the last stages of their sickness, they stood on the floor of the cage doubled up and appearing to suffer pain. Some died doubled up; others were found on their backs dead. In several cases the birds, apparently recovered from the first attack, began to hop around and eat again, but a second attack soon followed, and, in every case, this proved fatal.

<sup>1</sup> *Ztschr. f. Hyg. d. Haustiere*, 1908, 4, p. 33.

<sup>2</sup> *Jour. Med. Res.*, 1908, 18, p. 277.



Autopsy showed the spleen swollen. In several cases, when the blood vessels were cut, gas bubbled out. In four or five cases, this happened when the vessels were cut before the death of the animals. Drops of blood taken from a small vein under the wing, one to two hours before death, revealed under the hanging drops small, actively motile bacilli which appeared to be in pure culture. Smears were made from blood taken from the vein of the wing and stained. Microscopical examination of the stained smears showed the absence of all other organisms.

Smears made from the heart's blood on slant agar and in broth gave pure cultures. Out of 23 birds which died, pure cultures of a specific bacillus were obtained from 15 and the same organism was obtained by plating out from a mixture of bacteria in the other cases. The bodies of the latter had stood so long before the examination of them that a post-mortem invasion of the body had taken place. In four cases, cultures made from the blood taken from a branch of the humeral vein and obtained before the death of the animal gave pure cultures of the organism.

**Description of the bacillus.**—The organism is a small, very actively motile bacillus (0.7 to 1.5  $\mu$  long), rounded on the ends. It grows well at 37° C., and at room temperature, both under aerobic and anaerobic conditions, the latter tests being made according to Buchner's method. The bacillus stains well with ordinary stains, but is Gram-negative. No capsules or involution forms were noted.

**Cultural characteristics.**—Smears made on agar slants gave an abundant spreading, raised, translucent, glistening, smooth growth. Older cultures become slightly opalescent.

On potato, an abundant, spreading, raised, slimy growth is produced. The medium is darkened. Gelatin is liquefied in from 9 to 12 days when kept at 22° C. Broth becomes uniformly cloudy in 18 hours. Litmus milk is rendered slightly acid in 24 hours. The acidity increases slowly until the milk is coagulated, which takes from six to eight days. The coagulation takes place very slowly, the milk becoming thicker and thicker until a solid mass is formed. The milk has a slight aromatic odor.

The production of gas in glucose agar is profuse. In almost every case, the agar was found split up and the plugs blown out of the tubes in 24 hours; rarely did it take as long as 48 hours for this to take place.

In lactose agar, the growth follows the needle-track; but the production of gas is variable. In some cases, gas has been observed, but in most cases, gas was not produced when sugar-free beef extract had been used in making the agar.

On agar plates, the bacilli appear as small white pin-head colonies.

In 24 hours, the reaction in glucose-litmus-agar plates is acid, the plates being completely red; but, in from two to three days, the plates again become alkaline. The reaction in lactose-litmus plates remains alkaline.

In dextrose fermentation tubes, 100 per cent of gas is invariably produced in 48 hours. The gas ratio,  $H:CO_2$ , is 1:2. Gas production in lactose fermentation tubes varies. If gas is produced at all, it is produced very slowly and in small quantities. In a number of cases, a small amount of gas has been noted in from 48 to 72 hours. Galactose, maltose, saccharose, mannite, and levulose are all readily fermented.

In order to trace more accurately the change of the reaction in dextrose-litmus agar, 100 c.c. of dextrose broth were placed in a small flask and sterilized. After sterilization, the broth gave a neutral reaction. This broth was inoculated with the organism, incubated at 37°, and titrated every 12 hours for four days.

The acidity of dextrose broth inoculated with bird organism was as follows:

Hours:	0	12	24	36	48	60	72	84	96
Per cent:	0	1.6	2	2.2	2.4	1.8	1.2	0.8	0.15

These results show an increase of acid up to 48 hours; but a gradual decrease thereafter until the fourth day when the reaction becomes almost neutral to phenolphthalein or alkaline to litmus. This phenomenon is possibly due to the fact that the organism attacks the carbohydrates much more readily than the proteins. When the carbohydrates are used up, the proteins are then attacked and  $NH_3$  is split off. This neutralizes the acid formed from the fermentation of the sugars. Probably the two reactions are going on simultaneously, but the formation of acid is so much faster than the liberation of  $NH_3$  during the first 48 hours that the latter reaction is masked.

**Pathogenicity.**—One c.c. of 24-hour broth culture of the bacillus was injected into the pectoral muscle of a pigeon. The bird showed effects in about 24 hours. It refused to eat, stood on the floor of the cage, and revealed a number of symptoms noted in the sparrows. It lost weight continually for about five days. It then gradually recovered and again became active. During its illness, the excretions were very watery and consisted mostly of mucus. A small loopful of this mucus-like substance was transferred to a melted glucose-agar tube: this was poured into a sterile petri dish and incubated at 37° for 24 hours. From the large number of small white colonies which appeared on the plate, bacilli which gave all the cultural reactions of the bacillus found in the sparrows were fished out. A second pigeon was inoculated with a like amount of the same organisms. The effects were the same in both cases.

Since the excretions of the pigeons were not examined for the bacillus before inoculation, it is not certain that it is not present normally in the intestinal tract of the birds. The great number found on the plates would seem to indicate this. The diarrheal discharge may have been induced by some toxic substance produced by the injected bacteria. The excretions became normal again when the symptoms disappeared. Three sparrows were inoculated and these died in from two to three days after inoculation. Since all the sparrows which had died previously succumbed in from two days to two weeks after being placed in the cages, it is difficult to conclude that the death of those inoculated was due to the injection.

Three guinea-pigs were inoculated subcutaneously with 2 c.c. of a suspension of a 24-hour agar culture of the bacillus in physiological-salt solution. In two days, one developed an induration at the point of inoculation. The other two either gave negative results or the effects were too slight to be noticed.<sup>1</sup> The bacillus was recovered from the serous exudate after four days and cultivated on nutrient agar. A second guinea-pig was inoculated subcutaneously with 2 c.c. of a suspension of a 24-hour agar culture of the recovered bacillus. In two days it developed a large induration which

<sup>1</sup> Six animals had been inoculated with the bacillus before these but all gave negative results.

became soft and broke on the third day. The bacillus recovered from the second animal was passed through a third guinea-pig and the bacillus obtained from an induration produced in this animal was tested on all the ordinary media and it agreed with the original bacillus. This bacillus was used in the experiments that follow:

Two guinea-pigs, weighing 290 gm. each, were inoculated intraperitoneally with 1 c.c. of a suspension of a 24-hour agar culture. Both died in less than 18 hours. Animals inoculated with smaller doses lost in weight for a time, but recovered. Animals inoculated subcutaneously with 2 c.c. of a suspension of the recovered bacillus all developed indurations at point of inoculation, in two cases of which the bacillus was again recovered. No attempt was made to obtain the organism in the other two cases. In all fatal cases, the bacillus was recovered from the purulent exudate in the peritoneal cavity, and in two cases it was recovered from exudates in the pleural cavity and from the heart.

Guinea-pigs which were inoculated both subcutaneously and intraperitoneally with the original organism all recovered.

A rabbit, weighing 1,590 gm., was inoculated intraperitoneally with 2 c.c. of a suspension of a 24-hour agar culture from an induration. The next morning it was found doubled up in the corner of the cage. When agitated, it refused to move and did not eat when fed. It seemed to be extremely weak. In 48 hours it had lost 200 gm. in weight. The loss in weight continued until the fourth day when the animal moved about a little, but still seemed weak and stupid. On the fifth day, it ate when fed and began to move about more actively. From this time on, a gradual recovery was noted.

All the organisms recovered from the dead animals and those recovered from indurations were tested on all the ordinary culture media. The reactions varied little from those given by the original culture.

From a careful study of the morphology, cultural reactions, and even the pathogenicity of this bacillus, the resemblance to bacilli of the proteus group was evident. In order to study this resemblance more carefully and also to compare its reactions with those of other intestinal organisms, comparative tests were made with stock cultures of *B. proteus vulgaris*, *B. zenkeri*, *B. proteus mirabilis*, *B. cloacae*, *B. paratyphosus*, *B. enteridis*, *B. coli*, and the *B. cholerae suis*. The bacillus under study resembled in both morphological and cultural characteristics the stock culture of *B. cloacae* (Jordan) except that it is more actively motile. It differs from *B. coli* in being more actively motile and in producing more gas in 24 hours than is usually produced in the same length of time by *B. coli* and in the liquefaction of gelatin. The time necessary for the coagulation of milk is also much longer for the bacillus isolated from birds than for *B. coli* and the gas ratio is reversed.

**Agglutination.**—Two healthy rabbits were used in the immunization experiment. These animals were immunized by means of suspensions of the bacillus, isolated from

the sparrows, in physiological-salt solution. Inoculations were made subcutaneously every day for eight successive days, then every other day for three weeks. The initial dose was 1 c.c. of a suspension of the bacilli in sterile physiological-salt solution which had been heated at 65° C. for five minutes. This was followed on the second day with 1 c.c. of an unheated suspension of the same organism. The doses were gradually increased until 4 c.c. of an unheated suspension of the bacilli were injected at one time. The animals were anaesthetized and 5 c.c. of blood were taken from the heart in each case. This was centrifugalized and the serum was used in the agglutinating experiments.

The bacillus found in the birds was agglutinated by the immunized rabbit serum in dilutions as high as 1:50,000 in four hours. The stock culture of *B. cloacae* was agglutinated completely in 20 hours in dilutions of 1:100 and 1:200 but less completely in dilutions of 1:500 and 1:1,000, while *B. coli* and *B. vulgaris* were both completely agglutinated in dilutions as high as 1:50 but less completely in dilutions of 1:100 and 1:200.

If the cultural and morphological characteristics of the bacillus isolated from sparrows in this laboratory be compared with the reactions described by a number of previous observers as characteristic of organisms isolated from the blood and organs of birds during an epidemic among these animals, a striking resemblance will be noted. In some cases, these bacteria were described as *B. coli* or coli-like organisms; in other cases, where the differences were more marked or the study made more carefully, new names were given. A careful examination of the cultural reactions of the coli-like organisms as described by some observers shows many variations from the *B. coli* type. If, however, these reactions be compared with the cultural characteristics of *B. cloacae* the resemblance is so marked that one is inclined to believe that these observers were studying a bacillus of the proteus group and not a variety of *B. coli*. Since I have demonstrated that the bacillus isolated from the birds, which had died from an infection produced by it, can be rendered pathogenic, not only for pigeons, but for rabbits and guinea-pigs as well, and that this organism seems to be in all respects identical with that first isolated by Jordan from sewage and called by him *B. cloacae*, one is inclined to think, at least, that *B. coli* has been accused of producing many diseases for which *B. cloacae* is responsible. Since both organisms are found in the intestines of animals and hence in sewage, together with the fact that they resemble each other very closely in cultural reactions, it is probable that the differences between these two bacilli may have been overlooked by some observers.

## SUMMARY AND CONCLUSION.

1. A bacillus isolated from the blood of sparrows during an epidemic among these birds was the probable cause of their death, since it was obtained in pure culture from all that died and similar symptoms were produced in pigeons inoculated with it.

2. By passing this bacillus through animals, its virulence was increased so that it became pathogenic for guinea-pigs and rabbits.

3. So far as can be determined from the description given by other observers this bacillus resembles very closely certain bacteria isolated by them in bird epidemics and called by them *B. coli* or coli-like organisms.

4. The bacillus found in sparrows gives all the cultural characteristics of *B. cloacae* (Jordan) a member of the proteus group.

5. The serum of rabbits injected with the bacillus found in the sparrows will agglutinate the homologous organism in dilutions as high as 1:50,000.

The writer takes pleasure in thanking Dr. E. O. Jordan for his suggestions in this work.



## THE OPSONIC INDEX IN ACUTE ARTICULAR RHEUMATISM.\*

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A NUMBER of observers maintain that they have found a specific micro-organism in acute articular rheumatism. That acute articular rheumatism is a specific disease produced by a specific organism is claimed by Triboulet and Apert, Westphal, Wassermann and Malkoff, Fritz Meyer, Poynton and Paine, Beaton and Walker, Beattie, Lewis and Longcope, and others. This claim is based upon the fact that in a number of cases of this disease a micrococcus has been isolated which produces in rabbits and monkeys polyarthritis, endocarditis, and other manifestations of acute articular rheumatism. The observers admit that this organism, *M. rheumaticus*, cannot be differentiated morphologically or culturally from *Strept. pyogenes*. It is only by the production of the disease in animals that it shows its specificity. For example, Beattie<sup>1</sup> finds that inoculation into rabbits of *M. rheumaticus* produces a non-purulent arthritis in 60 per cent and endocarditis in 33 per cent while *Strept. pyogenes* causes a purulent arthritis in 18 per cent and endocarditis in only 2 per cent of the animals.

On the other side Cole<sup>2</sup> maintains that "arthritis and endocarditis may be produced by the intravenous inoculation of rabbits with streptococci from various sources, and the results obtained are quite similar to those described as resulting from the inoculation of the so-called *Micrococcus* or *Diplococcus rheumaticus*." He considers it unwarranted to establish a distinct variety of streptococci based on this property of causing endocarditis and arthritis.

Meakins<sup>3</sup> has recently corroborated the findings of Cole, producing non-purulent arthritis by the intravenous inoculation of rabbits with various strains of streptococci.

Inasmuch as the specific opsonic index in certain acute infectious

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<sup>1</sup> *Brit. Med. Jour.*, 1906, 2, p. 1781.

<sup>2</sup> *Canad. Jour. Med. and Surg.*, 1909, 25, p. 71.

<sup>3</sup> *Jour. Infect. Dis.*, 1904, 1, p. 714.

diseases follows a definite and typical course, it was thought that it might prove of interest to determine the course of the index to *M. rheumaticus* and streptococci in acute articular rheumatism.

Two strains of *M. rheumaticus* were employed. One was kindly furnished by Professor Beattie, the other came from the laboratory of Poynton and Paine.

An effort was made to isolate similar streptococci from the patients under observation in order to determine the opsonic index to the homologous organism. Blood cultures were made in three of the most severe cases in broth and milk and broth slightly acidified with lactic acid as suggested by Poynton and Paine.<sup>1</sup> The cultures all remained sterile.

Philipp,<sup>2</sup> Cole,<sup>3</sup> Beattie,<sup>4</sup> Marchildon,<sup>5</sup> and others also failed to obtain positive results from blood cultures. Cultures of the joint exudate (by Dr. Irons) in one case of my series proved sterile. This also agrees with the results of the writers just mentioned. However, Loeb<sup>6</sup> isolated a streptococcus from the blood in seven out of 45 cases of acute articular rheumatism, from a joint of one case, and from the blood of one patient with chorea, the nine strains corresponding culturally and morphologically to those described by Triboulet and Apert, Wassermann, Poynton and Paine, but he failed to produce with them arthritis in rabbits. Loeb believes it unlikely that the strains isolated by him are identical with those cultivated by Wassermann, Poynton and Paine, and others.

Meyer<sup>7</sup> and Menzer<sup>8</sup> isolated from the throats of patients with acute rheumatism streptococci which in rabbits produced multiple arthritis and often vegetative endocarditis. Hence throat cultures were made in eight of my cases, streptococci being isolated by means of blood-agar plates, human, rabbit, and sheep blood giving similar results. In one case the number of green colonies and hemolysing colonies of streptococci were about equal in number. The plates from two other cases showed three or four hemolysing colonies, the rest, several thousand, being green. In the remaining five cases the plates showed green colonies only. These results differ somewhat from those of Dr. D. J. Davis<sup>9</sup> who found an increase in the number of hemolysing streptococci in the throats of all of the cases of acute articular rheumatism he examined. Occasionally there was seen slight hemolysis around the greenish colonies, the corpuscles remaining intact immediately around the colony, as is frequently seen in 48-hour pneumococcus colonies. A greenish-brown discoloration in blood-agar cultures of *M. rheumaticus* has been described by Shaw<sup>10</sup> and by Walker.<sup>11</sup> The two strains of this organism studied by me (Beattie, Poynton and Paine) also produced similar green colonies in plates with human, rabbit, and sheep blood. The Poynton and Paine organism sometimes showed slight hemolysis around the green colonies in 48-hour plates.

Greenish color has been shown to be produced by pneumococci (Schottmüller,<sup>12</sup> Rosenow<sup>13</sup>) and by various strains of streptococci (Schottmüller), called by the latter *Strept. viridans*. The pneumococcus is differentiated from *Strept. viridans* by its

<sup>1</sup> *Practitioner*, 1901, 66, p. 22.

<sup>2</sup> *Deut. Arch. f. klin. Med.*, 1903, 76, p. 150.

<sup>3</sup> *Jour. Infect. Dis.*, 1904, 1, p. 714.

<sup>4</sup> *Brit. Med. Jour.*, 1906, 2, p. 1781.

<sup>5</sup> *St. Louis Med. Rev.*, 1908, 57, p. 185.

<sup>6</sup> *Arch. Int. Med.*, 1908, 2, p. 266.

<sup>7</sup> *Deut. med. Wchnschr.*, 1901, 27, p. 81.

<sup>8</sup> *Ibid.*, 1901, 27, p. 97.

<sup>9</sup> Personal communication.

<sup>10</sup> *Jour. Path. and Bact.*, 1904, 6, p. 158.

<sup>11</sup> *Brit. Med. Jour.*, 1907, 1, p. 1233.

<sup>12</sup> *Munch. med. Wchnschr.*, 1903, 50, p. 000.

<sup>13</sup> *Jour. Infect. Dis.*, 1904, 1, p. 308.

power to ferment inulin. Now, the two cultures of *M. rheumaticus* and the eight strains of streptococci producing green colonies on blood-agar plates, isolated from the throat in rheumatism, did not ferment inulin.

Ruediger<sup>1</sup> found green-producing colonies in all normal and nearly all diseased throats. He considers that they belong to a large group of organisms between typical pneumococci and streptococci. Some seem to correspond to Schottmüller's *Strept. viridans*, but others are closely related to the pneumococcus. He believes that all of these organisms including the *Strept. viridans* are closely related to one another and can be sharply differentiated from *Strept. pyogenes* by the following characteristics:

1. The cocci in smears made from milk cultures stain rather poorly and unevenly with Löffler's methylene blue, are usually somewhat elongated or lance-shaped, and often are found in pairs. *Strept. pyogenes* takes the methylene-blue stain readily, the cocci are never distinctly grouped in pairs in the chains and are not elongated, but frequently appear to be disc-shaped.

2. On ascites-agar slants a large proportion of these atypical organisms form lance-shaped or oval cocci which are grouped chiefly in pairs, but also in short chains.

3. When pure cultures are grown on serum-glucose-agar slants (three parts of glucose agar to one part ascites fluid or serum) no change is produced in the medium while *Strept. pyogenes* turns it white and opaque in from 36 to 48 hours. According to Libman the pneumococcus produces no such change.

Smears from milk cultures of the strains of green-producing cocci isolated by me from the throat in rheumatism showed faintly staining chains in six of the eight cultures. None showed any elongation of the cocci. The other two strains, the strains of *M. rheumaticus* and *Strept. viridans* from a normal throat, stained perfectly distinctly, as did the strains of *Strept. pyogenes* (one being obtained from the throat in a case of rheumatism). On ascites agar three of the six strains of green-producing colonies from the throat in rheumatism showed oval or lance-shaped cocci. The three other strains and *M. rheumaticus*, *Strept. viridans* (normal throat), and *Strept. pyogenes* were indistinguishable morphologically.

On ascites-glucose agar the strains of *Strept. pyogenes*, *M. rheumaticus*, and three strains of the green-producing cocci produced much opacity. The other green-producing strains and *Strept. viridans* (normal throat) produced only slight opacity. Rosenow found that strains of pneumococci after isolation acquire the ability to cause opacity in serum-glucose agar. This proved true of two of four of the pneumococcus cultures that I tested. It would seem, then, that this means of differentiation cannot be considered conclusively accurate. Although these strains of streptococci show differences morphologically, just as they do culturally in their growth in milk, these are not sufficient to differentiate them. None of the green-producing organisms correspond exactly to the classification of Ruediger. The two strains causing slight opacity in ascites-glucose agar, staining faintly in smears from milk and appearing as lance-shaped cocci on ascites-agar slants, come the nearest to corresponding to the organisms described by Ruediger.

Gordon and Houston<sup>2</sup> and later Andrews and Horter<sup>3</sup> have classified streptococci according to their different chemical reactions on various media. According to Andrews and Horter a strain of *M. rheumaticus* from Paine is classified as *Streptococcus salivarius*; one from Beattie as *Streptococcus faecalis*. The authors find that

<sup>1</sup> Jour. Infect. Dis., 1906, 3, p. 755.

<sup>3</sup> Ibid., 1906, 2, pp. 708, 775, 852.

<sup>2</sup> Lancet, 1905, 2, p. 1400.

"*Streptococcus salivarius* passes by insensible gradations into *Streptococcus faecalis*." They believe that Schottmüller's hemolyzing *Strept. pyogenes* corresponds to theirs, and that his *Strept. viridans* probably corresponds in general to their *Strept. salivarius* and *Strept. faecalis*. According to this classification the strains of *Strept. viridans* isolated by me from throats of rheumatism patients would belong to the same class as the strains of *M. rheumaticus* of Paine and Beattie.

Meakins<sup>1</sup> has examined eight strains of *M. rheumaticus* according to this method and finds that it "is not a distinct organism separate from other streptococci."

Beattie<sup>2</sup> states that *M. rheumaticus* can be differentiated from other streptococci by the production of acid and the precipitation of bile salts in McConkey's lactose broth. Meakins tested 25 strains of streptococci from different sources as to their reaction with this medium and found that the reaction was not peculiar to streptococci isolated from rheumatic patients.

Walker<sup>3</sup> finds that the streptococcus isolated from cases of rheumatism produces considerably more formic acid than is produced by streptococci from other sources. This might be a means of differentiation, but, as Cole points out, this test which is a quantitative one will probably not be of value in the study of an organism as variable in its characteristics as the streptococcus.

The only characteristic which seems to differentiate the green-producing streptococci isolated by me and the strains of *M. rheumaticus* (Beattie, Poynton and Paine) from *Strept. pyogenes* is the production of green colonies in blood-agar plates. On account of this property and their not fermenting inulin they may be considered as belonging to the streptococcus viridans group and in the rest of this paper the organisms isolated by me will be called *Strept. viridans* to distinguish them from the *M. rheumaticus* (Beattie, Poynton and Paine).

#### OPSONINS IN ACUTE ARTICULAR RHEUMATISM.

The opsonic index to various organisms has been estimated in the usual way in 18 cases of typical acute articular rheumatism. Ten were examined usually every other day during the course of the disease. In eight the index was observed only twice, the patients coming under observation at the end of the disease. The patients were all hospital cases. With one exception they had been ill from one to three weeks before entrance. However, in three cases (Charts 3, 4, 5) new joints became involved after admission, so that the indices could be estimated under these circumstances. The indices were taken to *M. rheumaticus* (Beattie, Poynton and Paine), *Strept. pyogenes*, *Strept. viridans*, and the pneumococcus. The opsonic index to the *Staph. aureus* and *Strept. viridans* (normal throat) were also estimated in the first six cases.

<sup>1</sup> *Canad. Jour. Med. and Surg.*, 1900, 25, p. 71.

<sup>2</sup> *Brit. Med. Jour.*, 1906, 2, p. 1781.

<sup>3</sup> *Brit. Med. Jour.*, 1907, 1, p. 1233.

In the charts *M. rheumaticus* refers either to the Beattie or to the Poynton and Paine organism. The two organisms were

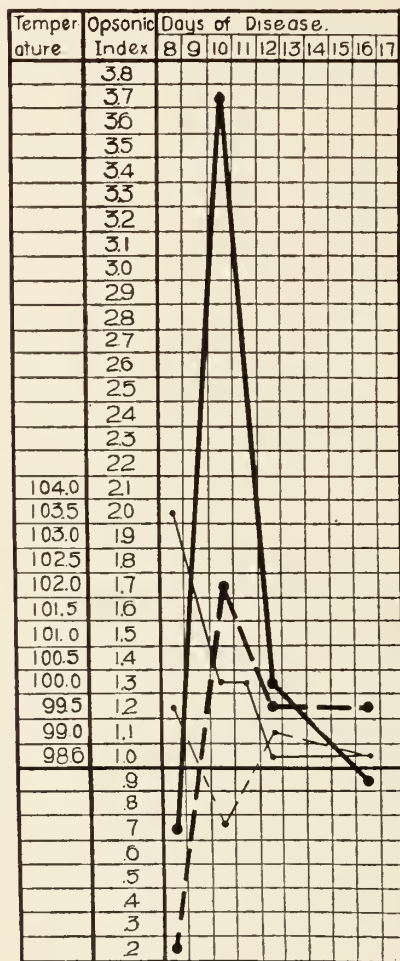


CHART 1.—ACUTE ARTICULAR RHEUMATISM (WOMAN, AGE 50).

Heavy solid line = Opsonic index to *M. rheumaticus*.

Heavy broken line = " " " *Strept. pyogenes*.

Fine broken line = " " " *Pneumococcus*.

Fine solid line = Temperature.

frequently both used and gave similar results. The strains of *Strept. pyogenes* were isolated from scarlet fever and erysipelas patients. Often two or more strains were employed but the opsonic index was the



same to the various strains, as I found to be the case also in my work on the index in scarlet fever and erysipelas. The homologous *Strept.*

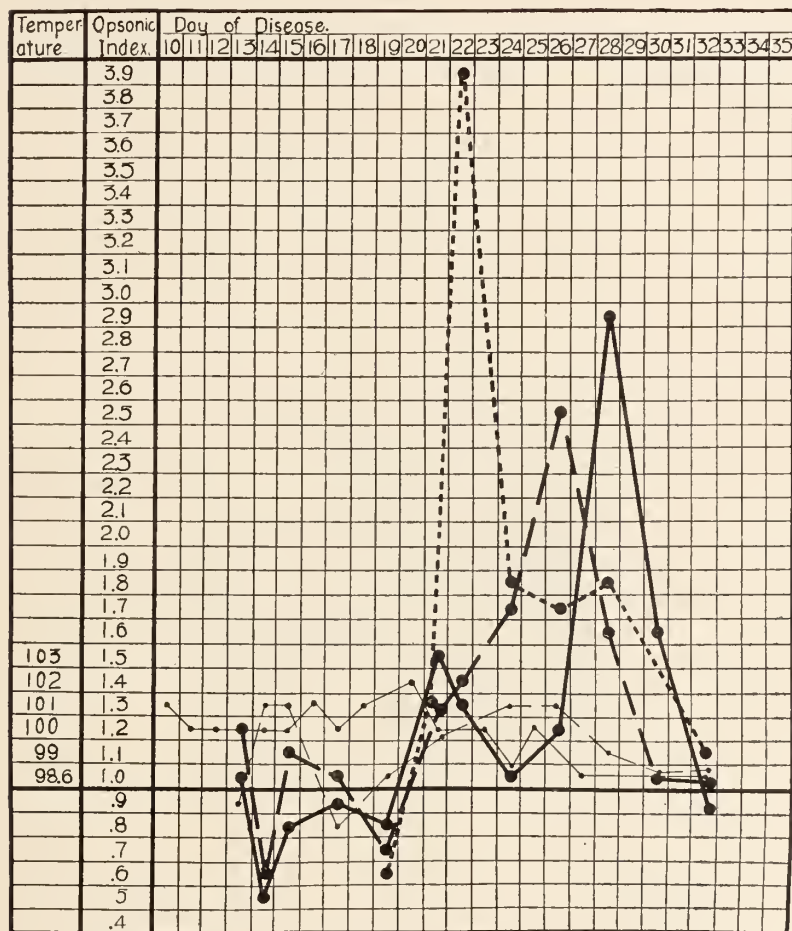


CHART 2.—ACUTE ARTICULAR RHEUMATISM AND ENDOCARDITIS (WOMAN, AGE 20).

Heavy solid line = Opsonic index to *M. rheumaticus*.

Heavy broken line = " " *Strept. pyogenes*.

Fine broken line = " " *Pneumococcus*.

Heavy dotted line = " " Homologous *Strept. viridans*.

Fine solid line = Temperature.

*viridans* was employed in five cases, in one case (Chart 6) both the homologous *Strept. viridans* and the homologous *Strept. pyogenes*.

The opsonic index to the strains of *Strept. viridans* isolated from

the throat in rheumatism was frequently estimated in other patients and the index closely corresponded to the homologous index. It was often difficult to obtain a sufficiently smooth suspension with the freshly isolated bacteria before the recovery of the patient. On this account and because the bacteria are frequently not phagocytal until grown on artificial culture media for several generations, the

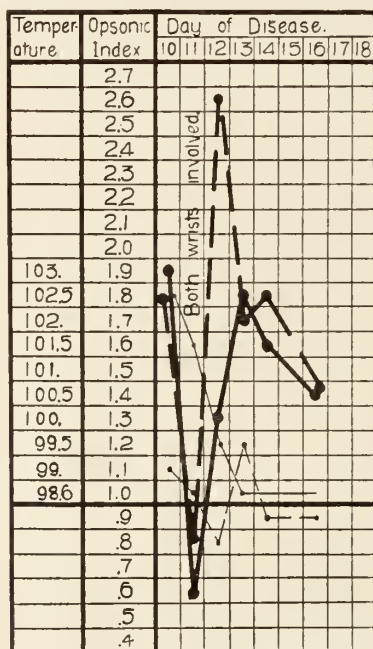


CHART 3.—ACUTE ARTICULAR RHEUMATISM (MAN, AGE 40).

Heavy solid line = Opsonic index to *M. rheumaticus*.

Heavy broken line = " " " *Strept. pyogenes*.

Fine broken line = " " " *Pneumococcus*.

Fine solid line = Temperature.

homologous organisms could not always be employed. It was found that the opsonic index followed the same course in the case of *M. rheumaticus* (Beattie, Poynton and Paine), *Strept. pyogenes*, and *Strept. viridans* (from patients with rheumatism) while it remained within the normal limits with respect to *Staph. aureus*, and, except in one case, pneumococcus and *Strept. viridans* from normal throat.

In the five cases examined before improvement commenced the opsonic index for *M. rheumaticus*, *Strept. pyogenes*, and

*Strept. viridans* (rheumatism) was found below normal—0.2–0.5 (Charts 1 and 2). With the involvement of new joints the index to these strains would fall to 0.5–0.7 (Charts 3, 4, 5). In all of the 18 cases just before the fall in temperature and improvement in the joints the opsonic index for the three strains of streptococci rose above normal, the average being 2.5. The charts show that the

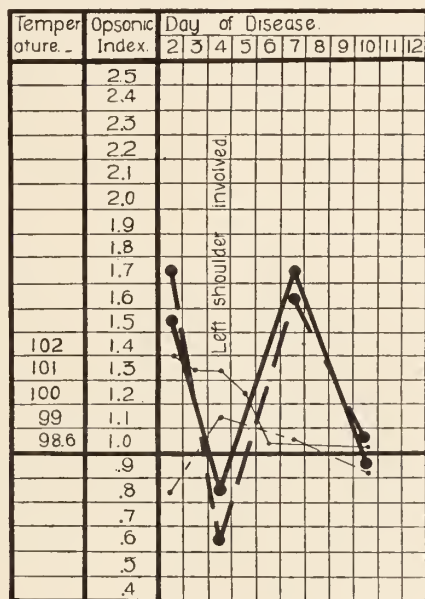


CHART 4.—ACUTE ARTICULAR RHEUMATISM AND ENDOCARDITIS, FOURTH ATTACK (MAN, AGE 35).

Heavy solid line = Opsonic index to *M. rheumaticus*.

Heavy broken line = " " " *Strept. pyogenes*.

Fine broken line = " " " *Pneumococcus*.

Fine solid line = Temperature.

indices for *M. rheumaticus*, *Strept. pyogenes*, and *Strept. viridans* (rheumatism) follow the same course and are often identical. In Chart 2 the difference perhaps appears greater than it really is, the indices all being above normal during about the same period. In one case (Chart 5) there was an increase in opsonin for pneumococcus and *Strept. viridans* (normal) but not coincidentally with the rise in the opsonic index for *M. rheumaticus* and *Strept. pyogenes*. No evident explanation could be found for this rise, the patient being carefully examined for pneumococcal complications. These results

agree with those obtained by me<sup>1</sup> in studying the opsonic index in scarlet fever. Here, too, the opsonic index for *Strept. viridans* (normal) followed the course of the pneumococcus and not that of *Strept. pyogenes*.

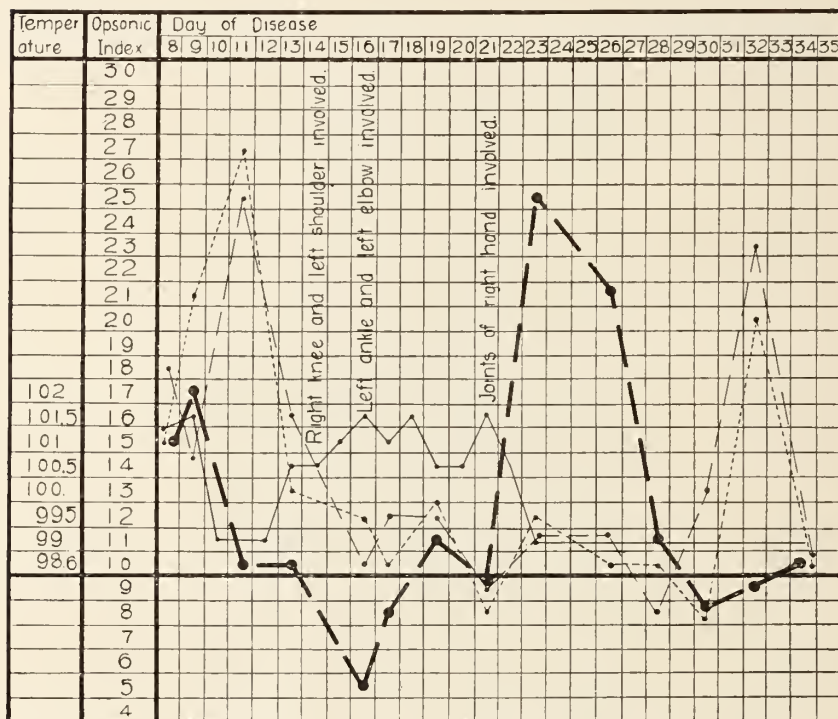


CHART 5.—ACUTE ARTICULAR RHEUMATISM (MAN, AGE 30).

Heavy broken line=Opsonic index to *Strept. pyogenes*.

Fine broken line = " " " Pneumococcus.

Fine dotted line = " " " *Strept. viridans* (Normal throat).

Fine solid line = Temperature.

In one case (Chart 6) from the throat of which both *Strept. pyogenes* and *Strept. viridans* were isolated, the indices for both these organisms correspond to the indices for *Strept. pyogenes* (scarlet fever) and *M. rheumaticus*. The study of the opsonic index in acute articular rheumatism to streptococci producing green colonies on blood-agar plates would suggest that this group of organisms isolated from the throat in rheumatism is closely related to the *Strept. pyogenes*.

<sup>1</sup> *Jour. Infect. Dis.*, 1907, 4, p. 304.

The opsonic indices in these experiments have been estimated in the usual way. From time to time the results have been controlled both by heating the normal and immune (patient's) serum to  $46^{\circ}$  for 15 minutes, and by diluting the serum to the point of opsonic extinction, or more correctly the point exceeding spontaneous

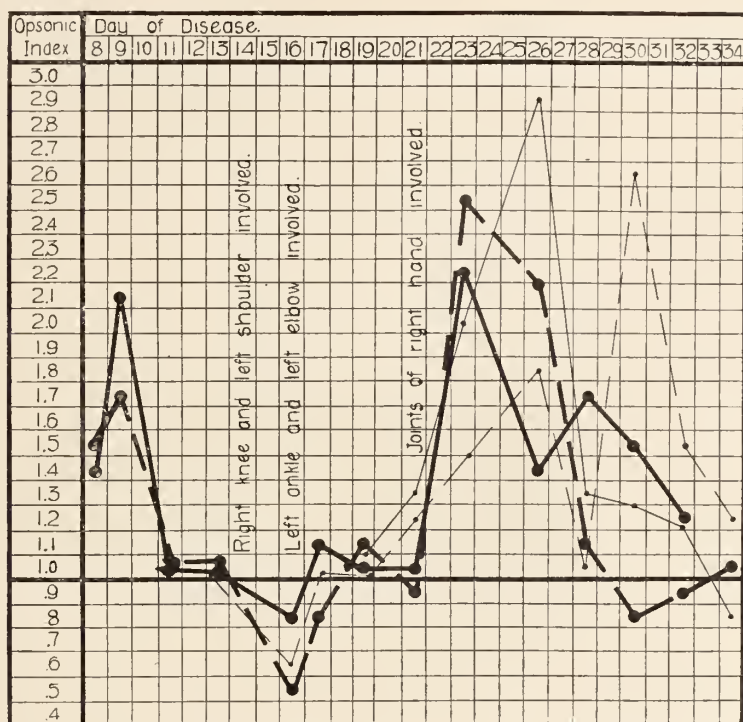


CHART. 6.—ACUTE ARTICULAR RHEUMATISM (MAN, AGE 50). (Same patient as in Chart 5.)

Heavy solid line = Opsonic index to *M. rheumaticus*,  
 Heavy broken line = " " " *Strept. pyogenes*.  
 Solid fine line = " " " Homologous *Strept. viridans*.  
 Broken fine line = " " " " *Strept. pyogenes*.

phagocytosis. For example, the opsonic indices obtained in the usual way for *M. rheumaticus* and *Strept. pyogenes* were 1.8 and 1.7; after heating the normal and immune serum to  $46^{\circ}$  for 15 minutes the indices were 2.7 and 2.8 respectively. With an index for *M. rheumaticus* of 1.4 with undiluted serum, on diluting the serum; the point of opsonic extinction for the normal was 1:24 while that of the immune serum was 1:94. One patient had an index of



0.6 for *Strept. pyogenes* determined in the usual way. On dilution of the serum, the point of opsonic extinction for the normal serum was 1:12 while that of the patient's serum was 1:6. Two days later with an index of 3.4 for the same organism the point of opsonic extinction for the normal serum was found to be 1:24 while that of the patient's was 1:94.

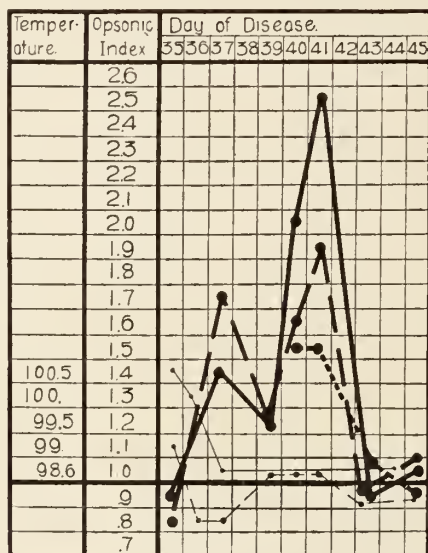


CHART 7. ACUTE ARTICULAR RHEUMATISM (WOMAN, AGE 40)

Heavy solid line = Opsonic index to *M. rheumaticus*.  
 Heavy broken line = " " " *Strept. pyogenes*.  
 Heavy dotted line = " " " Homologous *Strept. viridans*.  
 Fine broken line = " " " Pneumococcus.  
 Fine solid line = Temperature.

These results correspond in general with those obtained in diphtheria and erysipelas in which I also found that the course of the indices determined by heating and by diluting the serum corresponded closely with that obtained by the Wright method.<sup>1</sup> Klien<sup>2</sup> and Meakins<sup>3</sup> found that when, by the usual method of determining the opsonic index, they obtained normal indices, diluting the serum to the point of opsonic extinction brought out great differences between the

<sup>1</sup> *Jour. Infect. Dis.*, 1908, 5, pp. 14 and 238.

<sup>3</sup> *Jour. Exp. Med.*, 1900, 2, p. 100.

<sup>2</sup> *Johns Hopkins Hosp. Bull.*, 1907, 18, p. 245.

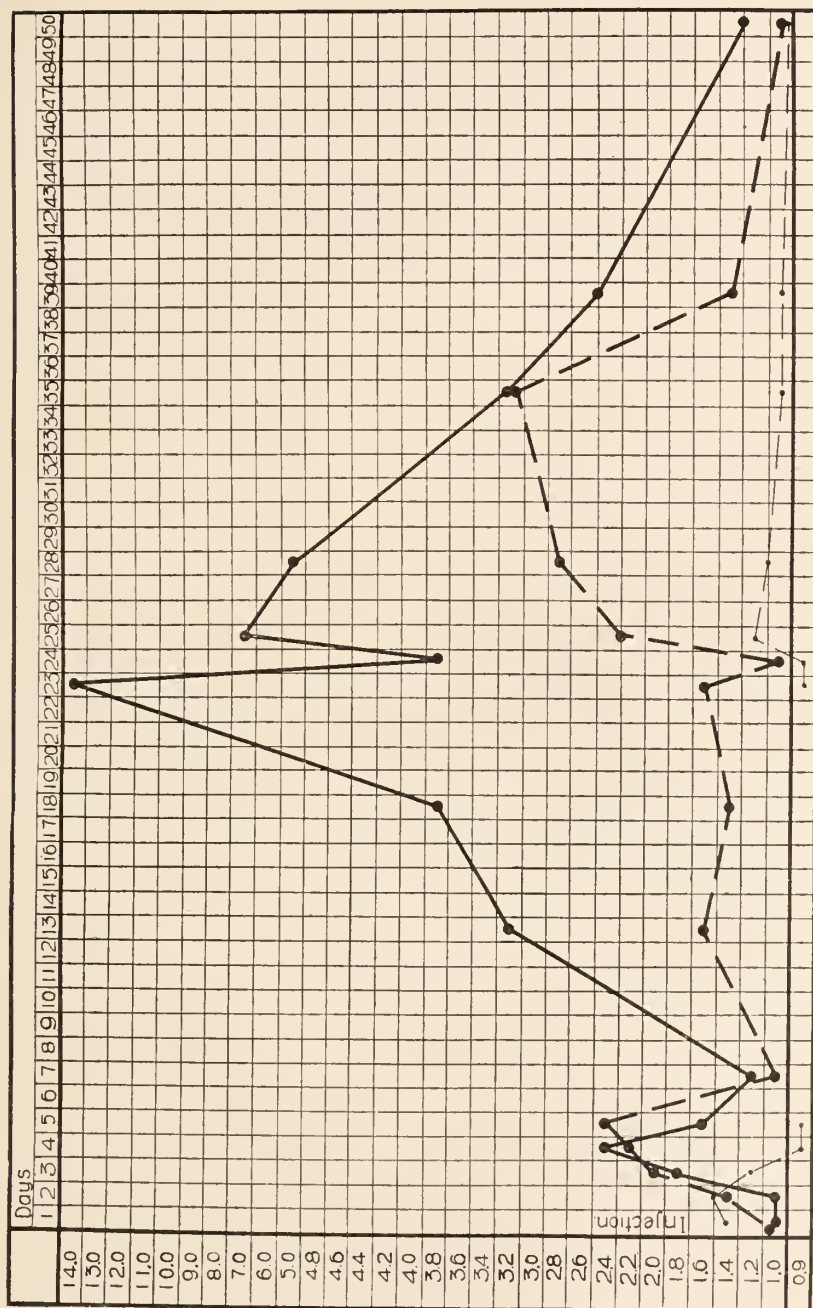


CHART 8.—OPSONIC CURVES OF RABBIT INJECTED INTRAVENOUSLY WITH 24-HOUR GROWTH OF FOUR BLOOD-AGAR STANTS OF *M. rheumaticus* (Beutlie).

Heavy solid line = Opsonic index to *M. rheumaticus*.

Fine broken line = Opsonic index to *Pneumococcus*.

Heavy broken line = " " " *Strept. pyogenes*.

normal and immune sera. It is possible the thickness of their suspension was such that accurate counts with the undiluted serum were not possible.

A rabbit (Chart 8) was injected intravenously with a 24-hour growth of four blood-agar slants of *M. rheumaticus* (Beattie). The animal was slightly sick the day after the injection, but no arthritis developed. The opsonic index was taken at first for *M. rheumaticus*, *Strept. pyogenes*, pneumococcus, staphylococcus, *Strept. viridans* (normal, and from rheumatism three strains); later only for *M. rheumaticus*, *Strept. pyogenes*, and pneumococcus. The index for the pneumococcus, staphylococcus, and *Strept. viridans* (normal) remained within the normal variations. The indices for the other streptococci all closely corresponded in their course. The differences in the height of the indices for *M. rheumaticus* and *Strept. pyogenes* after the 12th day is owing probably to the virulency of *M. rheumaticus* (Beattie). I have found that in immunized animals great differences in the height of the index, but not in the course, may occur, if a virulent and non-virulent organism of the same strain are employed, the differences being due to the small opsonic effect of the normal serum on the virulent strain. From Chart 8 it is seen that the serum of the rabbit immunized with *M. rheumaticus* shows an increase in opsonin for both *M. rheumaticus* and *Strept. pyogenes*.

Agglutination experiments were made with the serum of the immunized rabbit and that of 12 rheumatic patients. The experiments were repeated three or four times during the course of the disease. In each case both *Strept. pyogenes* and *M. rheumaticus* and in one case the *Strept. viridans* (homologous) were employed. All of the tests were macroscopic. At first calcium broth cultures were used according to the method of Hiss,<sup>1</sup> the tubes being incubated for three hours and then placed in the ice chest for 18 hours. Later 0.2 per cent dextrose broth cultures was employed after centrifugalization for a few minutes to remove clumps. The tubes were incubated for three hours and then left at room temperature for 18 hours.

The agglutination experiments were often unsatisfactory and had

<sup>1</sup> *Jour. Exper. Med.*, 1905, 7, p. 560.

to be repeated on account of the spontaneous agglutination of the organisms in the broth cultures. No more agglutinin was found in the immune rabbit serum than in normal. Of the 12 patients examined, agglutinins to both *Strept. pyogenes* and *M. rheumaticus* were demonstrated in seven. It is probable that if the serum of the other five patients had been examined oftener, agglutinins would have been found, as they were present as a rule on one day only in the positive cases. During the negative phase of the streptococco-opsonic indices the agglutinins were normal or below, normal serum agglutinating both strains of streptococci at a dilution of about 1:10. With the rise in the opsonic indices, the agglutinative power increased in four cases, the highest dilution at which agglutination occurred being 1:100. In one case the increase in agglutinins occurred the day before and in two cases the day following the rise in the opsonic indices. The homologous *Strept. viridans* was also tested in one case. It was agglutinated at a dilution of 1:100 at the height of the opsonic indices and continued to be so agglutinated for seven days.

Meyer<sup>1</sup> found that streptococci are agglutinated by specific immune serum and he differentiates, by means of agglutination, the streptococci of anginal, scarlatinal, rheumatic infections from those of simple pyogenic infections. He also found gradual differences in the agglutination of the anginal streptococci which were interpreted as speaking against their identity. Aronson,<sup>2</sup> however, does not find that a differentiation can be made between groups of streptococci by agglutination. Wlassjewski<sup>3</sup> found the serum from a case of rheumatism had no effect upon any of the streptococcus cultures employed (scarlet fever, puerperal fever, erysipelas, phlegmon, and dysentery). Weaver<sup>4</sup> has found that the agglutination reaction between streptococci cultivated from cases of scarlatina and the serum from cases of scarlet fever is in no way specific, these streptococci being agglutinated at about the same dilution by sera from cases of lobar pneumonia, erysipelas, and typhoid fever, and to almost the same extent by puerperal-fever serum.

<sup>1</sup> *Deut. med. Wchnschr.*, 1902, 28, p. 751.

<sup>2</sup> *Ibid.*, 1903, 29, p. 439.

<sup>3</sup> *Centralbl. f. Bact.*, Abt. I, 1903, 33, p. 464.

<sup>4</sup> *Jour. Infect. Dis.*, 1904, 1, p. 91.

## CONCLUSIONS.

In acute articular rheumatism the opsonic index for *M. rheumaticus* (Beattie, Poynton and Paine) and *Strept. pyogenes* followed the same course. With involvement of new joints and rise in temperature the indices for both these organisms fell below normal. With improvement in the joints and symptoms the indices rose above normal.

The opsonic index for *Strept. viridans* isolated from the throat in acute rheumatism followed the same course as the index for *M. rheumaticus* and *Strept. pyogenes*.

The indices for *Staph. aureus*, pneumococcus, and a strain of *Strept. viridans* from a normal throat remained within the normal limits.

The opsonic index consequently does not help to differentiate *M. rheumaticus* from *Strept. pyogenes*.

A rabbit immunized with *M. rheumaticus* gave an increase in opsonin for *M. rheumaticus*, *Strept. pyogenes*, and *Strept. viridans* (rheumatism), but none for *Staph. aureus*, pneumococcus, and *Strept. viridans* (normal).

Agglutinins to both *Strept. pyogenes* and *M. rheumaticus* were demonstrated in the serum of seven of 12 rheumatism patients, the course of the agglutinins corresponding in general to that of the opsonic indices.

From the results of this study the conclusion seems warranted that streptococci play an essential part in acute articular rheumatism.



# CONCERNING SO-CALLED AGGLUTINOIDS.\*

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THE agglutination reaction was first described as a specific reaction by Gruber and Durham in 1896. It has since been the subject of much investigation, which need not be recapitulated here. German investigators soon noticed that in making tests with serial dilutions agglutination might fail to appear at certain dilutions, although distinct at higher dilutions. These portions of a serial dilution where the agglutination failed to appear have been called inhibition areas (*Hemmungszonen*), and following Ehrlich a supposititious substance has been regarded as the cause of this failure, much as the ancients were inclined to assume special causes for all the different phenomena of nature. The body which prevents the expected action of the agglutinin is called "agglutinoid."

I have studied some antityphoid sera, which showed these phenomena nicely (Table 1). In this table, *s*=a slight reaction; +=a

TABLE 1.

ANIMAL	DATE		DILUTION OF SERUM											
	Bleeding	Test	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:5,000	1:10,000	1:20,000
			1:40,000	1:80,000	1:160,000	1:320,000	1:640,000	1:1,280,000	1:2,560,000	1:5,120,000	1:10,240,000	1:20,480,000	1:40,960,000	1:81,920,000
Sheep No. 56.....	Dec. 11	Dec. 11	o	s	c	c	c	c	+	c	o	c	c	o
Sheep "Stub".....	10	10	o	o	o	+	+	c	c	c	c	c	c	o
Black goat.....	10	10	o	o	o	o	s	c	c	+	c	c	+	o
Bay horse.....	4	10	+	+	+	s	s	c	c	c	c	c	c	o
Sorrel horse.....	10	10	c	c	c	o	o	o	o	+	c	c	+	o

distinct reaction; *c*=a complete reaction, that is, the liquid is cleared and all the germs settled to the bottom.

The serum from Sheep 56 did not agglutinate in a dilution higher than 1:320 and in low dilutions, as 1:10, agglutination was incomplete and in 1:5 entirely absent. In the serum from Sheep "Stub," agglutination was obtained in dilutions as high at 1:20,000, and the inhibitory area also extended farther up, so that complete agglutina-

\* Received for publication May 10, 1909.

tion is first seen in the dilution 1:160. In the serum from the black goat, although the final dilution is only 1:20,000, agglutination is first seen at 1:640. It will readily be seen that should a typhoid patient possess a serum like this, the ordinary clinical methods of performing the agglutination test would not detect it. In the serum from the bay horse, the final dilution extends to 1:80,000 and now some agglutination occurs in the lower portion of the inhibition area. In the serum from the sorrel horse, we have the same phenomenon except that here the agglutination is complete and perfect in the low dilutions, absent for a space, then again complete.

Some experiments have been made to throw light if possible on the cause of these phenomena. Table 2 shows the tests made to

TABLE 2.

SERUM	DILUTION OF SERUM											
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:5,000	1:10,000	1:20,000	1:40,000
Antityphoid	c	c	o	o	o	o	c	c	c	c	c	+
Inactivated 56° C. ½ Hr.	c	c	o	o	o	o	c	c	c	c	c	+
Antityphoid ½.....	c	c	+	o	o	o	c	c	c	c	c	+
Normal horse ½.....	c	c	+	o	o	o	c	c	c	c	c	+
Antityphoid ½.....	l	l	o	o	o	o	c	c	c	c	c	+
Guinea-pig ½.....	l	l	o	o	o	o	c	c	c	c	c	+
Water ½.....	c	c	+	c	c	c	o	o	o	o	o	o
Normal horse ½.....	c	c	+	c	c	c	o	o	o	o	o	o

determine the influence, if any, that the presence or absence of complement might exert. The results show that neither the entire absence of complement (heated at 56° C. for one-half hour) nor an excess of complement (either from horse or guinea-pig) made any appreciable difference in the results. In the test with guinea-pig serum *l* indicates complete lysis of the germs.

TABLE 3.

ANIMAL	DATE		DILUTION OF SERUM										
	Bleed- ing	Test	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:5,000	1:10,000	1:20,000
			1:40,000	1:80,000	1:160,000	1:320,000	1:640,000	1:1,280,000	1:2,560,000	1:5,120,000	1:10,240,000	1:20,480,000	1:40,960,000
Sorrel horse	Dec. 17	Dec. 18	c	o	o	o	s	s	c	c	c	c	c
Goat No. 76	15	18	o	o	+	+	+	+	c	c	c	+	c
Goat No. 76	16	18	o	o	+	+	+	+	c	c	c	+	+
Goat No. 76	17	18	o	o	+	+	+	+	c	c	c	+	s

The animals yielding these sera were being injected twice a week, and I tested the serum from one animal daily to determine what effect, if any, the injection might have. Table 3 shows no appreciable change.

I now turned to a study of the effect, if any, that the age of the culture might have. Table 4 shows that the age of the culture affects the

TABLE 4.  
AGE OF CULTURE.

CULTURE		DILUTION OF SERUM														
		1:12½	1:25	1:50	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200	1:102,400	
Grown on agar	{ 15 hrs. . .	3	4	5	5	3	2	1	0	0	0	0	0	0	0	
	{ 24 hrs. . .	5	5	5	6	6	5	4	2	2	1	1	0	0	0	
Grown in broth	{ 15 hrs. . .	3	3	4	5	4	3	1	1	0	0	0	0	0	0	
	{ 24 hrs. . .	4	5	5	5	4	3	1	0	0	0	0	0	0	0	
	{ 5 days . .	5	5	5	3	5	5	4	4	4	4	2	1	0	0	

agglutinating power, a 24-hour culture on agar being agglutinated in twice as high a dilution as a 15-hour culture, while a five-day broth culture is similarly more agglutinative than a 24- or 15-hour culture. The inhibition area is, however, more marked in the younger cultures.

In these tests the degree of agglutination is indicated by the numerical system: the higher the number, the more complete the agglutination. In interpreting an agglutination reaction, there are two factors to be considered: (1) the granules, floccules, or sediment; and (2) the cloudiness or clearness of the fluid. Of course, these phenomena are inversely proportional to each other and I have found that recording each separately enables one to indicate differences that otherwise would escape being recorded. We may distinguish five degrees of each and indicate them by numbers from 0 to 4, as shown:

Flocculation	Number	Cloudiness	Old System
None	0	complete	no reaction
Slight	1	abundant	slight
Distinct	2	distinct	+
Abundant	3	slight	+
Complete	4	none	complete

In recording a reaction both the flocculation and the residual cloudiness are recorded independently and then added.

The results with the age of the cultures, shown in Table 4, seem to indicate that some substance produced during the growth of the

germs and which tends to accumulate is connected with the agglutination reaction. The slower diffusion in agar might account for the more rapid accumulation of this substance in agar cultures.

Some have contended that agglutination is really produced by a precipitation, the germs being caught and carried down in the precipitate. Now, if this hypothetical substance, which thus favors agglutination, is outside the germ body, we should be able to wash it away. To test this, I have removed the growth from an agar culture with physiological-salt solution, emulsified thoroughly with a mechanical shaker, and recovered the bacilli by centrifugalization; these were again emulsified in salt solution and allowed to stand 20 hours. At the end of 20 hours, the bacilli were recovered by the centrifuge, again emulsified, again recovered, and then made up into a suspension of the usual density and used at once. The physiological-salt solution used in all this work contained 0.4 per cent of formaldehyde to prevent germ growth. Table 5 shows that agglutination occurred as usual and that the hypothetical substance could not be removed by such means.

If the substance were in the germ body, it might possibly be removed by the plasmolytic action of distilled water; hence, I repeated the washing as before, except that distilled water was used in place of physiological-salt solution. Table 5 shows that practically no

TABLE 5.  
EFFECT OF WASHING.

	DILUTION OF SERUM									
	1:12½	1:25	1:50	1:100	1:200	1:400	1:800	1:1600	1:32,000	1:64,000
NaCl sol .....	4	5	5	6	6	5	4	3	3	1
Distilled water .....	0	0	0	0	0	0	0	0	0	0

agglutination occurred. However, this did not show that I had removed from the germ bodies a hypothetical something necessary to agglutination, for upon adding salt to these tubes they agglutinated as usual. I had merely shown that in some way salt is necessary to agglutination.

Many other tests of various kinds were made without any illuminating results. However, in working with different suspensions from day to day, it was found that the density of the suspension influences the character of the reaction. Table 6 shows this distinctly. It will

TABLE 6.

DILUTION OF EMULSION	DATE		DILUTION OF SERUM														
	Bleed- ing	Test	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:5,000	1:10,000	1:20,000	1:40,000	1:80,000	1:160,000	
1:4	Dec.	Dec.	+	o	o	o	s	s	c	c	c	c	c	+	+	o	
1:2	17	22	+	+	+	+	+	c	c	c	c	+	+	+	+	o	
1:1	17	22	+	+	+	c	c	c	+	+	+	+	+	+	o	o	

be observed that a total inhibition area occurs only in the dilute suspension. Further, that, while the final limit of agglutination is not greatly changed, the limit of complete reaction is distinctly changed,

TABLE 7.

DILUTION OF SUS- PENSION	DILUTION OF SERUM											
	1:12½	1:15	1:50	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600
1:32	8	0	0	0	0	4	4	8	8	8	6	0
1:16	8	2	2	4	4	8	8	8	8	6	2	0
1:8			6	4	8	7	4	4	4	4	1	0
1:4	6	6	6	8	7	6	6	4	4	4	2	0
1:2	6	6	7	8	5	4	3	3	3	1	1	0
1:1	7	8	8	8	6	3	2	2	2	1	0	0

being lower in the denser suspensions. The point of beginning complete reaction is also lowered, so that the effect is to lower or push

TABLE 8.

NUMBER	HgCl <sub>2</sub>	KI	WATER	RESULTS	FINAL DILUTION	
					HgCl <sub>2</sub>	KI
1	1 C.C. n/10	1.0 C.C. n/1	0.0 C.C.	—	n/20	n/2
2	"	0.4 "	0.6 "	—	"	n/5
3	"	0.16 "	0.84 "	+	"	n/12½
4	"	1.0 C.C. n/10	0.0 "	+	"	n/20
5	"	0.4 "	0.6 "	+	"	n/50
6	"	0.16 "	0.84 "	S	"	n/125
7	"	1.0 C.C. n/100	0.0 "	—	"	n/200
8	"	0.4 "	0.6 "	—	"	n/500
9	"	0.16 "	0.84 "	—	"	n/1,250



TABLE 9.

DILUTIONS OF HgCl <sub>2</sub>	DILUTIONS OF KI											
	5n	2n	$\frac{4}{5}n$	$n/2$	$n/5$	$n/12\frac{1}{2}$	$n/20$	$n/50$	$n/125$	$n/200$	$n/500$	$n/1,200$
n/200....	+	+	+	+	+	+	+	+	+	+	+	+
n/20.....	+	+	+	+	+	+	+	+	+	+	+	+
n/2.....	+	+	+	+	+	+	+	+	+	+	+	+

TABLE 10.

Suspension of typhoid bacilli .....	DILUTION OF SERUM								
	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280
	o	+	c	c	c	c	+	o	o
Suspension of staphylo- cocci.....	DILUTION OF HCl								
	n/1	n/2	n/4	n/8	n/16	n/32	n/64	n/128	n/250
	o	+	c	c	c	c	c	+	o

TABLE 11.  
(BUXTON AND RAHÈ.)

DILUTIONS OF MASTIC	DILUTIONS OF Fe(OH) <sub>3</sub>								
	1:10	1:500	1:1,000	1:2,000	1:3,000	1:5,000	1:8,000	1:16,000	1:20,000
1:300	-	-	-	-	-	+	+	Tr	-
1:200	-	-	-	-	-	+	+	Tr	-
1:120	-	-	-	-	+	+	+	-	-
1:60	-	-	-	+	+	+	-	-	-
1:10	-	-	+	+	+	-	-	-	-

back the area of complete reaction in the denser suspensions. Table 7 shows a similar result when using a greater range of densities and the numerical notation. Now, this phenomenon is important in that it is comparable to well-known chemical phenomena: when bichloride of mercury and potassium iodide are mixed in certain proportions, the result shown in Table 8 is obtained. Here the bichloride, corresponding to the emulsion, maintains a constant dilution ( $n/20$ ), while the KI, corresponding to the serial dilution of serum, varies from  $n/2$  to  $n/1,250$ . A precipitate occurs or the reaction is positive only within certain limits ( $n/12\frac{1}{2}$  to  $n/125$ ), as is the case in agglutination. In the chemical reaction we say the precipitate is soluble in excess of either reagent, whatever this may mean. In agglutination we do not know what to say. Now, when the concentration of the bichloride is varied, we get the same lowering or pushing back of the area of positive reaction as in agglutination (see Table 9).

Again, various chemicals will agglutinate germs. Note in Table 10 the resemblance between the agglutination with hydrochloric acid and the agglutination with a specific antiserum. In agglutination with the acid the germs must be suspended in a salt-free medium in contrast to the serum which requires the presence of salt. In agglutination with the acid one sees an inhibition area as well marked as with the serum.

It must be admitted that from hydrochloric acid and the bichloride of mercury to serum is a long cry and the value of these resemblances might be questioned; but in the flocculation of colloids we encounter a phenomenon much more nearly resembling the agglutination of germs and which may serve as a connecting link between them.<sup>1</sup>

In Table 11 it will be seen that when colloidal mastic is flocculated with colloidal  $\text{FeCl}_3$ , varying the dilution of the mastic causes the zone of reaction to be pushed back exactly as is the case with a suspension of germs or with bichloride of mercury.

Again, Table 12, also taken from Buxton and Rahè's article, shows that the flocculation of colloidal platinum with  $\text{FeCl}_3$ , of colloidal mastic with  $\text{FeCl}_3$ , of colon germs with  $\text{FeCl}_3$ , and of

<sup>1</sup> Buxton and Rahè, *Jour. Med. Research*, 1909, 20, p. 113.

TABLE 12.  
(BUNTON AND RAHÈ.)

	Dilutions Results	n/1 +	n/10 +	n/100 +	n/500 —	n/5,000 —	n/6,000 +	n/10,000 +	n/12,000 +	n/20,000 —	n/8,000 —	n/4,000 +
Colloidal platinum and FeCl <sub>3</sub>												
Mastic and FeCl <sub>3</sub>	Dilutions Results	n/1 +	n/20 +	n/40 —	n/500 —	n/1,000 —	n/2,000 +	n/3,000 +	n/4,000 +	n/6,000 +	n/3,000 +	n/4,000 +
<i>B. coli communis</i> FeCl <sub>3</sub>	Dilutions Results	n/1 —	n/20 +	n/200 +	n/300 +	n/400 —	n/600 —	n/1,000 —	n/1,500 +	n/2,000 +	n/3,000 +	n/4,000 +
<i>B. typhosus</i> and serum	Dilutions Results	1:5 +	1:10 +	1:50 +	1:40 —	1:320 —	1:640 +	1:1,280 +	1:5,000 +	1:20,000 +	1:80,000 +	1:160,000 —

typhoid germs with a specific antiserum is of the same type throughout, and shows a well-marked inhibition area.

It is thus shown that the peculiar inhibition areas found in agglutination with a specific antiserum are not unique, but that similar phenomena can be traced down through organic colloids and inorganic colloids to simple inorganic chemistry. Hence, although we do not yet understand the cause of the phenomena, we are no longer justified in assuming the existence of a special causative agent—the so-called “agglutinoids.”

# THE BACTERIAL INTEGRITY OF CELLOIDIN AND PARCHMENT MEMBRANES.\*

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## INTRODUCTION.

THE celloidin membrane occupies an important place in the bacteriological technique of today because of its perfect osmotic properties, and its accepted imperviousness to the passage of bacteria. The osmotic properties are well known. The bacterial integrity for all micro-organisms has been assumed from the results of isolated experiments with certain bacteria, which were found not to pass through the membrane. It is the purpose of this paper to give the results of experiments made with the object of testing the bacterial integrity of the celloidin, and also of the parchment membrane, to various types of bacteria, especially to the intestinal group.

**Historical.**—The celloidin sac was introduced for bacteriological purposes by Morpurgo and Tirelli,<sup>1</sup> who exploited it as a ready means for the cultivation of the tubercle bacillus. Their method was to place a piece of infected tissue into a celloidin sac, which was then sealed and placed either under the skin or preferably into the peritoneal cavity of a rabbit. They found that the sac soon filled with cell-free serum, and in several weeks there appeared an abundant growth of the tubercle bacillus. Sacs were allowed to remain in the peritoneal cavity of rabbits upward of two months without any of the rabbits showing signs of infection. This demonstrated the impermeability of the celloidin membrane for the tubercle bacillus.

Metchnikoff, Roux, and Salimbeni<sup>2</sup> further advanced the use of the celloidin sac in their study of the toxin production of the cholera spirillum. They filled celloidin sacs with inoculated broth, and introduced them into the peritoneal cavity of guinea-pigs. The animals succumbed on the third to the fifth day, with all the signs of cholera poisoning. At autopsy, the sacs were found intact and no cholera spirilla could be demonstrated in the peritoneal fluid, heart's blood, or viscera.

Two years later Nocard and Roux<sup>3</sup> ingeniously used the celloidin sac in the demonstration of the cause of pleuro-pneumonia of cattle. The results of these investigators, in the successful cultivation of an almost ultra-microscopic organism, by the use of the celloidin sac, seemed to be ample as evidence confirming the conclusions of Morpurgo and Tirelli, and Metchnikoff, Roux, and Salimbeni that the celloidin membrane offers

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<sup>1</sup> *Archives ital. de biologie*, 1892, 18, p. 187; *Centralbl. f. Bakt., Ref.*, 1893, 13, p. 74.

<sup>2</sup> *Ann. de l'Inst. Pasteur*, 1896, 10, p. 257.

<sup>3</sup> *Ibid.*, 1898, 12, p. 240.

an impermeable barrier to bacteria as well as to body cells, while it permits of perfect osmosis.

Nocard also used the sac in his experiments on the transformation of the human into the avian type of tubercle bacillus, by placing the bacilli in celloidin sacs inserted into the peritoneal cavity of a fowl. He found that the tubercle bacilli did not pass through the sac wall.

Up to this time the celloidin sac had been used for intraperitoneal placement only. Carnot and Fournier were the first to use it in tube experiments, made in their study of the pneumococcus and its toxins. They found that the pneumococcus occasionally past through the sac as evidenced by turbidity in the outside fluid.

McCrae<sup>2</sup> used the sacs in a study of the agglutinins obtained with paratyphosis and enteritidis cultures. To control his technic of making the sacs he incubated them, after inoculation, for 24 hours in broth. Finding no turbidity in the outside fluid, he held that the sacs were intact, and ready for intraperitoneal placement. That the bacteria did not escape from the sacs, when in the peritoneal cavity of the rabbit, he held to be shown by the observation that upon the insertion of the inoculated sacs agglutinins appeared and gradually increased. On the removal of the sacs, they began to disappear.

Jordan, Russell, and Zeit<sup>3</sup> and Russell and Fuller<sup>4</sup> used the celloidin sac in an exhaustive study of the longevity of the typhoid bacillus. No definite experiments were conducted by these investigators with the object of testing the integrity of celloidin for the typhoid bacillus, since owing to the legal exigencies under which the experiments were made the results of previous experiments had to be accepted at their face value.

It thus appears that the basis for the belief of the bacterial integrity of the celloidin membrane rests upon experiments made, chiefly with the tubercle bacillus and the cholera spirillum. On the other hand the occasional turbidity found in the outside broth in the experiments of Carnot and Fournier, working with the pneumococcus, suggests either the presence of imperfections in the sacs, or that the pneumococcus could actually grow through the celloidin membrane.

**Technic.**—Novy<sup>5</sup> first described a method of making the celloidin sac. Previous investigators, Morpurgo and Tirelli and Metchnikoff, Roux, and Salimbeni, failed to describe their technic. The method of Novy consisted in repeatedly dipping a glass tube into a solution of celloidin and allowing it to dry when sufficiently coated. It was then carefully folded back on itself from the upper extremity and completely inverted. This gave a well-formed sac which was firmly attached by ligatures to a piece of glass tubing. After sterilization in broth the sac was inoculated, and closed by sealing the glass tube in the flame. Ruffer and Crendiropoulou,<sup>6</sup> and Eyre<sup>7</sup> changed the technic somewhat relative to the method of separating the celloidin from the glass tube. They dipped the coated tube alternately for a few seconds into strong alcohol and then into water. The water and alcohol diffusing through the celloidin gradually loosened it from the glass and the sac was readily stripped off. A glass tube was then inserted into the neck of the sac and fastened and closed by a method similar to that of Novy. Eyre strengthened the junction between the sac and glass tube by a ligature

<sup>1</sup> *Archiv. de méd. expér.*, 1900, 12, p. 357.

<sup>2</sup> *Jour. Exper. Med.*, 1901, 5, p. 635.

<sup>3</sup> *Jour. Infect. Dis.*, 1904, 1, p. 650.

<sup>4</sup> *Ibid.*, 1906. Supplement No. 2, p. 40.

<sup>5</sup> *Bacteriological Laboratory Handbook*, 1898.

<sup>6</sup> *Brit. Med. Jour.*, 1900, 2, p. 1305.

<sup>7</sup> *Bacteriological Technique*, 1902, p. 279.



before he sealed it. The sac was fixt in an empty tube, and the whole sterilized by dry heat at 150° C. Gorsline<sup>1</sup> molded sacs over a piece of tubing which had a small opening left in the end. The celloidin was then stript off by forcing water through the tube. Prudden<sup>2</sup> earlier used a similar method in which he forced air through the tube instead of water. The sac was attached to a glass tube by heating, and closed by sealing the glass in the flame.

McCrae reported the successful use of the gelatin capsule over which to mold the celloidin sac. His method was later modified by Harris, who used a piece of tubing with a constriction near the end which he fixt to the cap of the capsule by slightly heating the tubing, and bringing it into contact with the cap. The entire capsule was then dipt into celloidin and when sufficiently coated, was allowed to dry. The gelatin was melted by hot water and pipetted off, the sac being closed by sealing the glass tube in the flame.

In these various methods a piece of glass tubing remains as part of the sac and, when placed in the peritoneal cavity of the experimental animal, it acts more or less as an irritant. Harvey<sup>3</sup> has obviated this trouble by a further modification of the gelatin-capsule method of molding the celloidin sac. He made an opening into the body of the capsule large enough to permit the passage of a piece of glass tubing which was heated and attached to the inside of the cap. He then dipt the capsule into a paraffin bath allowing an even coat of paraffin to harden on the outside of the capsule, which was then dipt into celloidin until of sufficient thickness. When the last layer had set, the whole was dipt into chloroform, which dissolved the paraffin and left the gelatin capsule free from the celloidin. The sac was then dipt into alcohol and then into water so that the gelatin was softened and could be readily withdrawn, leaving the celloidin sac. This he closed by filling the neck with aseptic wool and covering it over with melted paraffin. The neck was then wiped dry with a piece of absorbent cotton soaked in alcohol, and sealed with celloidin.

The most serviceable technic for making any desired size of celloidin sac is the method described by Kellerman<sup>4</sup> and supplemented by Frost.<sup>5</sup> The method consists in pouring the celloidin solution into a glass tube of the desired size and, after the coating has air hardened, in shrinking it from the glass by pouring in water. This method is easy of application, rapid, and can be utilized to make a sac of any desired size.

In my work the method of Kellerman was followed. The celloidin used is an eight per cent solution by weight of equal parts of absolute alcohol and sulfuric ether.<sup>6</sup> A large tube served for molding, which gives a sac with a capacity of about 5 c.c. The celloidin is poured into the tube till about one-fourth full and, while rotating the tube, the celloidin is poured back into the stock bottle leaving an even coating free from bubbles. The tube is rotated for a few minutes in a horizontal position and then the celloidin is hardened by a current of air forced into the tube from a foot-bellows. After the celloidin is dry enough so that it does not stick to the finger, and before it has started

<sup>1</sup> *Science*, 1902, 15, p. 375.

<sup>2</sup> Cited by Harris, *Johns Hopkins Hosp. Bull.*, 1902, 13, p. 112.

<sup>3</sup> *Centralbl. f. Bakt.*, 1908, 46, p. 285.

<sup>4</sup> *Jour. Applied Micros.*, 1902, 5, p. 2038.

<sup>5</sup> *Amer. Pub. Health Assoc. Rep.*, 1902, 28, p. 536.

<sup>6</sup> It is important that these proportions be followed for if an excess of alcohol is used the sacs dry slowly and unevenly, while if too much ether is used the sacs dry too rapidly and are hard to handle because of cracking.

to shrink from the side of the tube, a second coating of celloidin is added as above. When dry, the sac is filled with water and removed by pulling on it and separating the celloidin from the wall of the tube by a dull instrument. It is fitted to a glass tube of slightly smaller bore, ligated with heavy thread, and sealed with celloidin. After this it is filled with water which prevents further drying. It was found that if the sacs were allowed to dry in the air until they were greatly creased and of a whitish color they readily broke and were unfit for use.

The celloidin sacs used in animal work were molded in the ordinary test-tube cut off to a length of two inches. Upon removal of the sac, a glass tube of 3 mm. bore was inserted, and the top of the sac drawn down tight with a wrapping of heavy thread, the ends being left long. This so fashioned the celloidin that when the glass tube was pulled out it left a sac with a small opening and a wide flange at the neck. This flange is readily flattened out and the edges cut off short. The sac is filled with water and dropped into a test-tube and in this condition can be kept in stock. For use the sac is filled, immersed in broth contained in a large test-tube, and sterilized. The sterile sac is withdrawn from the tube by means of the long thread and so held by an assistant.<sup>1</sup> A small amount of the broth is removed by means of a sterile pipette, and the broth in the sac inoculated by a loop of the desired culture. Care should be taken not to touch the sides of the sac during the process. After inoculation the sac is closed by pulling on the threads and tying. To seal, the sac is seized at the neck with a pair of sterile forceps, the water removed by absorbent cotton soaked in absolute alcohol, and several coatings of celloidin applied. The sac is repeatedly washed with sterile water and returned to the tube of broth ready for intraperitoneal adjustment.

#### GROWTH OF BACTERIA THROUGH THE CELLOIDIN MEMBRANE.

Experiments, as we have seen, have been made which are the foundation of the assumption of the integrity of the celloidin membrane for all bacteria. These experiments were with *B. tuberculosis* and *Sp. cholerae*. Other bacteria were not used, and it has been my purpose to find the status of the integrity of the celloidin membrane for other bacteria, principally those of the intestinal group.

Each sac, used in the following experiments, was tested for leaks by immersing it in water and blowing into the sac as hard as possible. If no leaks were detected by this air test, the sac was filled with a saturated egg-albumin solution (freshly prepared) and immersed up to the glass neck in as small an amount of water as possible contained in a 500 c.c. graduate, the sac being held in place by cotton packed around the glass tube. At the end of 48 hours the outside water was tested for the presence of albumin by the buiret and Millon tests. If these were negative the integrity of the sac was held established. The egg albumin was then carefully washed out and the sac was ready for use.

<sup>1</sup> A pair of needle forceps clamped to a ring stand is serviceable.

Treating the celloidin membrane with chloroform and alcohol seemed to make it harder to the touch. It is generally held that this treatment makes the membrane more durable. Accordingly, a series of sacs was hardened, after being air dried, with chloroform for five minutes followed by alcohol for five minutes. As to the durability the treated sacs, although they felt harder and seemed to be a little tougher than the untreated, they behaved in similar experiments exactly as did the latter.

The permeability of the treated and untreated sacs was tested with sodium chloride. Sacs were filled with distilled water containing enough added salt to make the chlorin test 140 parts per million. The content of the sacs was tested for chlorin at intervals as expressed in Table 1.

TABLE 1.  
CHLORIN FOUND IN CELLOIDIN SACS AFTER PERIODS IN SALT SOLUTION (140 PARTS PER MILLION).

	UNTREATED		TREATED	
	Series A	Series B	Series A	Series B
45 minutes.....	14.4	10.2	9.6	4.8
3 hours, 15 minutes.....	43.2	52.8	19.2	14.4
18 hours.....	43.2	67.2	43.2	48.0
24 ".....	52.8	115.2	91.2	91.2
31 ".....	100.8	124.8	96.0	96.0
43 ".....	120.0	115.2	115.2	115.2
52 ".....	120.0	120.0	124.8	135.4
72 ".....	124.8	135.4	129.6	135.4

From these results it appears that the variation in the time required to establish an equilibrium in the untreated and treated sacs is so slight that, as far as the permeability for the chlorides is concerned, the hardening of the sacs has little if any effect. One significant fact is brought out in the length of time required to establish an equilibrium. This speaks for the thickness of the celloidin sacs used which, according to the prevailing ideas, should offer an effectual barrier to the passage of bacteria.

To determine the bacterial integrity of the celloidin membrane, sacs were filled with broth and immersed in broth contained in ordinary commercial pepton bottles. Each sac was held in place by cotton well packed about the neck, and the tube of the sac also plugged with cotton. The outfit, container and sac, was then sterilized by heating in the autoclav for 10 minutes. Upon cooling, a

part of the broth in the sac was removed with aseptic precautions so that the broth in the sac was about 1 cm. below the junction of the celloidin and glass. Then the sac was raised so that the celloidin-glass junction was above the surface of the broth in the container. This procedure obviated the exposure to the growth of the bacteria of possible thin portions about the junction of the celloidin and glass. Each outfit, after sterilization and the necessary after-manipulations, was placed in the incubator for at least 24 hours and, if no turbidity was observed in the fluid either within or without the sac, it was ready for inoculation. The sac was inoculated with a loop of a 24-hour culture of the chosen micro-organism, and outfit was placed in the thermostat. Observations were made at frequent intervals for any turbidity in the outside broth. If turbidity was observed a small amount of the broth was withdrawn and plated in appropriate dilutions. Cultures were isolated from the colonies and the bacterium identified. In all cases where the outside broth became turbid the species of bacteria with which the sac had been inoculated, was isolated. The results are expressed in Table 2.

TABLE 2.  
GROWTH OF BACTERIA THROUGH CELLOIDIN MEMBRANE.

	Average Period of Incubation	Recovery from Outside Broth	Number of Experiments	Earliest Time of Recovery*
<i>B. prodigiosus</i> .....	24 hours	+	10	.....
<i>B. pyocyaneus</i> .....	3 days	+	2	.....
<i>B. coli</i> .....	24 hours	+	7	9 hours
<i>B. aerogenes</i> .....	24 hours	+	4	9 "
<i>B. chol. suis</i> .....	3 days	+	2	.....
<i>B. enteritidis</i> .....	3 "	+	2	.....
<i>B. paratyphosus</i> .....	3 "	+	2	.....
<i>B. typhosus</i> .....	24 hours	+	8	4 hours
<i>B. dysenteriae</i> .....	48 "	+	2	.....
<i>B. alkaligenes</i> .....	24 "	+	2	.....
<i>B. vulgaris</i> .....	36 "	+	2	.....
<i>B. cloacae</i> .....	24 "	+	2	.....
<i>Staphylococcus</i> .....	6 days	o†	6	.....
<i>Streptococcus</i> .....	6 "	o	5	.....
<i>Pneumococcus</i> .....	8 "	o‡	4	.....
<i>B. subtilis</i> .....	11 "	o	3	.....
<i>B. anthracis</i> .....	11 "	o	3	.....
<i>B. pseudodiphtheriticus</i> .....	13 "	o	5	.....
<i>Sp. cholerae</i> .....	20 "	o‡	8	.....
<i>Sp. metchnikovi</i> .....	20 "	o	2	.....

\* If a period before 18 hours.

† In one case a pure culture of staphylococcus was recovered from the outside broth on the fifth day. The air and albumin tests were negative.

‡ In one case a pure culture of the spirillum was recovered from outside broth. The air and albumin tests were negative.

§ Milk was used as a culture medium and not broth.

A study of these results demonstrates conclusively that certain species of bacteria, of which the intestinal group is a type, readily

grow through the celloidin membrane. On the other hand, the cocci—staphylococcus, streptococcus, and pneumococcus—the diphtheria group, the anthrax group, and the spirilla—of cholera and metchnikovi—do not usually grow through. There were two single exceptions in the case of the staphylococcus and the cholera spirillum. The results of the experiments with the members of the intestinal group and *B. prodigiosus* and *B. pyocyaneus*, show that the current view of the bacterial integrity of the celloidin membrane is not tenable for all bacteria. Some are able to grow through and others are not.

It would appear at first glance that the motility of the bacteria was a factor in the ability of certain micro-organisms to grow through the celloidin membrane. The cocci, non-motile, did not grow through, while *B. typhosus*, motile, readily grew through. However, *B. aerogenes*, non-motile, readily grew through and *Sp. cholerae*, very actively motile, did not grow through in 20 days. Thus the explanation does not lie in motility but rather appears to be a peculiar property of certain bacteria, motile or non-motile.

To show that the growth of the bacteria through the celloidin membrane was not because of leaks in the sacs, the following control was made. Each outfit, after the test of the growth of the bacteria in the sac had been completed, was sterilized in the autoclav, taking the precaution to have the sac well immersed in the broth. After sterilization the sac was removed from the container and tested for leaks by the air test. If this was negative the sac was filled with a saturated egg-albumin solution and immersed in water. If the air test was positive or if after four days the outside water showed the presence of albumin, the entire experiment was discarded. By this procedure there was an absolute check upon the results of the experiment as far as the detection of any defects present in the sacs which would permit of the ready passage of bacteria in any way than through the actual thickness of the celloidin membrane.

#### THE DIRECT PASSAGE OF BACTERIA THROUGH THE CELLOIDIN MEMBRANE.

Having found that certain bacteria were able to grow through the celloidin membrane, experiments were undertaken to learn, if



possible, whether this group of bacteria could pass directly through in the absence of a pabulum for growth.

This determination is of importance in view of the technic that was used by Jordan, Russell, and Zeit, and later by Russell and Fuller in the study of the longevity of the typhoid bacillus. Their method was to seed celloidin and parchment sacs with the typhoid bacillus and then expose the sacs in sewage or running water, examining the sacs at frequent intervals for the presence of typhoid bacilli. In accordance with the results of previous experimenters the bacterial integrity was assumed to hold in periods up to several weeks.

Johnson<sup>1</sup> questioned the bacterial integrity of the parchment membrane in the light of his work with parchment sacs at the Columbus Sewage Testing Station. He found that parchment sacs of initial and continued integrity, as far as dialyzing properties were concerned, permitted the escape of *B. coli* when immersed in distilled water. In flowing sewage large numbers of motile bacteria including *B. coli* passed through the sac wall in a comparatively short time. He did not experiment with the typhoid bacillus but says, "That if other less motile forms could pass through the walls of unpunctured parchment sacs under such conditions, there is no room for reasonable doubt, regarding the ability of the typhoid-bacillus to act in a similar manner." This observer did not experiment with celloidin.

The plan of my experiments to test the direct passage of the bacteria, was to use distilled water in container and sac, to sterilize the outfit in the autoclav, and to seed the content of the sac with a loop of the desired culture. This was very carefully removed from the agar-slant so as not to carry over any culture medium which would favor the growth of the bacteria. The outfit was then placed in a vessel of running tap-water by which a temperature was maintained ranging from 8°-12° C. This simulated, as far as temperature was concerned and its effect on the bacteria, the condition if the bacteria had been exposed directly in running water. Three species of bacteria were used—*B. typhosus*, *B. pyocyaneus*, and *B. prodigiosus*. Withdrawals of 5 c.c. of the outside water were made at intervals, run into broth, and incubated. If there was growth, plates were

<sup>1</sup> Jour. New Eng. Water Works Assoc., 1905, 10, p. 508

made, colonies picked and identified,<sup>1</sup> with the results as expressed in Table 3.

TABLE 3.  
DIRECT PASSAGE OF BACTERIA THROUGH THE CELLOIDIN MEMBRANE.

	AVERAGE SEEDING	TIME OF RECOVERY FROM OUTSIDE WATER		NUMBER OF EXPERIMENTS
		Earliest	Latest	
<i>B. prodigiosus</i> .....	540,000	16 hours	48 hours	10
<i>B. pyocyaneus</i> .....		27 "	60 "	3
<i>B. typhosus</i> .....	286,000	14 "	67 "	8

From this it is shown that *B. prodigiosus*, *B. pyocyaneus*, and *B. typhosus* are able to pass directly through the celloidin membrane and that, too, without an increase or growth in the sacs. In the experiments with *B. typhosus* the number of colonies found on the plates when 1 to 2 c.c. of the outside water was directly plated ranged from 20 to 50.

Not alone in experiments with a pure culture of these bacteria but in the presence of the bacteria found in tap-water were *B. prodigiosus* and *B. typhosus* found to pass readily through the celloidin membrane. Sacs were filled and immersed in tap-water but not sterilized. In experiments with *B. prodigiosus*, plates were made with appropriate dilutions to see if the water contained any red-pigment producers. If none were found the sacs were seeded with *B. prodigiosus* and if red colonies appeared on plates of the outside water, it was taken to show the passage of *B. prodigiosus* through the sac wall. Controls showed that there were no red-pigment producers in the tap-water kept in containers during the same period of time. In the case of *B. typhosus* the sacs were seeded and the outside water plated at intervals on Conradi-Drigalski medium. Typical colonies were picked at periods ranging from 46 hours to three days, and identified by cultural tests and the agglutination reaction (1:1000). These are positive results which further go to prove the power of certain bacteria to pass directly through the celloidin membrane, in the presence of other micro-organisms.

In my first experiments the method was to seed the celloidin sac

<sup>1</sup> The identification of *B. prodigiosus* and *B. pyocyaneus* was made from the cultural reactions and pigment-production. The identification of *B. typhosus* was made from the different culture tests and the agglutination reaction (dilution 1:1000).

and test the outside fluid for the presence of the bacterium. Experiments were now made to test the passage of bacteria from without into the sac. Russell and Fuller report negative results of experiments in which sacs were immersed in running sewage and tests made for the presence of sewage bacteria in the sacs.

Celloidin sacs, filled with sterile water, were immersed in fresh sewage contained in a large vessel, and the content of the sacs tested at frequent intervals for the presence of bacteria. A comparative study made of sacs filled with sewage and the outside water (sterile tap-water) tested for bacteria, and sacs filled with sterile water and immersed in sewage (Table 4) shows that the bacteria of the intestinal flora readily pass through the membrane, either from within outward or from without inward, the time required in the former condition being apparently somewhat longer than in the latter.

TABLE 4.  
PASSAGE OF SEWAGE BACTERIA THROUGH THE CELLOIDIN MEMBRANE.\*  
SEWAGE INSIDE OF SAC.

SERIES	TIME OF RECOVERY OF BACTERIA FROM OUTSIDE WATER		
	24 hours	48 hours	108 hours
I.....	+	....	....
II.....	o	+	....
III.....	+	....	....

SERIES	TIME OF RECOVERY OF BACTERIA FROM INSIDE WATER		
	24 hours	48 hours	108 hours
I.....	+	....	....
II.....	o	o	+
III.....	o	+	....

\* *B. alkaligenes* and *B. coli* were the predominant bacteria which passed through, as recovered from the plates.

A very striking result observed in the experiments was the ability of the typhoid bacillus to pass through the celloidin membrane. It was recovered from the outside broth as early as four hours after the sac was seeded. In the case of the colon bacillus the earliest period of recovery was nine hours. The difference in the penetrating power of the two bacteria was observed by Cambier<sup>1</sup> who found that the typhoid bacillus passed through the Chamberland bougie when

<sup>1</sup> *Comptes rend. de l'Acad. de sci.*, 1901, 132, p. 1442.

immersed in broth in a period of 18-20 hours, while *B. coli* and other forms took a longer period. This fact he made the basis of a method suggested for the isolation of the typhoid bacillus.

I carried out a small series of experiments using the idea of Cambier for the isolation of typhoid bacilli from stools, but instead of using the Chamberland bougie, used the celloidin sac.

Stools were obtained from patients having typical symptoms of typhoid fever and a positive agglutination reaction, and celloidin sacs, filled and immersed in sterile broth, were inoculated with a loop of the feces and incubated. Withdrawals of the outside fluid were made after the sixth hour after inoculation at intervals of two hours if possible. The object was to obtain the organism which first passed through the sac wall. The broth was plated in blood glucose agar, and a plan of identification followed, according to the system of Epstein.<sup>1</sup> From 27 samples of stools, of 10 different patients in the first to the third week of the disease, no typhoid bacilli were recovered. *B. alkaligenes* was found to be the predominant organism in about 70 per cent of the samples and an unidentified bacillus, which is very actively motile, gram-negative, and does not ferment the ordinary sugars in about 30 per cent of the samples (Table 5). *B. coli* was found to pass through in all cases. There is no doubt that typhoid bacilli were abundant in the stool but in the experiments probably did not pass through the celloidin membrane before other forms.<sup>2</sup>

TABLE 5.  
ISOLATION OF THE TYPHOID BACILLUS FROM FECES.

CASE	NUMBER OF SAMPLES	PREDOMINANT BACTERIUM		<i>B. coli</i> FOUND
		<i>B. alkaligenes</i>	Unidentified Bacillus	
1.....	3	3	...	3
5.....	2	2	...	2
6.....	2	...	2	2
7.....	3	1	2	3
38.....	3	3	...	3
40.....	2	1	1	2
44.....	4	4	...	4
60.....	1	1	...	1
71.....	3	...	3	3
72.....	4	4	..	4

<sup>1</sup> Amer. Jour. Med. Sci., 1908, 136, p. 190.

<sup>2</sup> Further work is to be carried on along somewhat different lines with the object of thoroughly testing the celloidin membrane as a basis of a method for the isolation of the typhoid bacillus from stools. I wish to take this opportunity to thank Professor E. R. Le Count for his many kindnesses shown and Drs. Rogers and Speidell of the Cook County Hospital for their services in obtaining the samples.

## THE BACTERIAL INTEGRITY OF PARCHMENT SACS.

This type of diffusible membrane is also held to be impervious to the passage of bacteria. Sacs were made out of the best parchment tubing obtainable, and sealed according to the method of Jordan, Russell, and Zeit. Many leaks were found in the sacs, it being almost impossible in a series of eighteen to find one that was negative to the albumin test. On testing, *B. prodigiosus* was found to pass readily through the parchment wall.

The non-dependable integrity of the ordinary parchment tubing was recognized by Russell and Fuller, and they used the diffusion shells of Schleicher and Schüll. I tested six of these shells, 38×85 mm. They were fitted to a glass tube of approximate bore, ligated and the junction sealed with celloidin. Each shell, upon testing, was negative to the air and albumin tests. They were then filled and immersed in broth. After sterilization they were seeded with *B. typhosus* and in each the outside broth was turbid at the expiration of 24 hours of incubation. A pure culture of *B. typhosus* was recovered. Each shell was sterilized and tested for leaks by the air and albumin tests and found negative.

These results clearly demonstrated that *B. typhosus* grows readily through the special parchment diffusion shells. Further, in view of the previous results with celloidin sacs, there is no room for doubt that the typhoid bacillus can also pass directly through the parchment shells in the absence of material for growth.

THE BACTERIAL INTEGRITY OF CELLOIDIN SACS IN THE  
ANIMAL BODY.

The extensive use of the celloidin sac for intraperitoneal placement in animal work has been largely in experiments with the tubercle bacillus and the cholera spirillum. The experimental results left no question but that the celloidin sac was impervious to the passage of these bacteria. The tube experiments with *Sp. cholerae*, cited in Table 2, further demonstrate the bacterial integrity of the celloidin membrane for this micro-organism. However, the bacterial integrity of the celloidin sacs for members of the intestinal group of bacteria in animal experimentation does not seem probable in view of my previous results.

McCrae,<sup>1</sup> working with *B. enteritidis* and *B. paratyphosus* in a study of their agglutinins, maintained that the sacs completely imprisoned the bacteria from the observation that the agglutinins fell when the sacs were removed. The question now arises, not as to the passage of agglutinogenic substance through the sac, but the probability of a slow escape of bacteria.

Sacs for intraperitoneal adjustment in rabbits were made as previously outlined. Their capacity ranged from 1½ to 3 c.c. The presence of leaks, it is readily seen, could not be determined by the air and albumin tests as was done in the case of the large sacs. However, an equally good test method was used. Twenty-four sacs were made and filled with broth. They were then divided into two lots. Each sac of one series was inoculated with staphylococci, which, it will be remembered, were found not to pass through the celloidin membrane (Table 2). The sacs were then sealed and returned to the tubes of broth and incubated. Each sac of the other lot was inoculated with *B. typhosus*, sealed, and returned to tubes of broth. Seven were incubated, and the remaining five were aseptically introduced into the peritoneal cavity of five rabbits.

Incubation of the 12 sacs inoculated with staphylococci showed growth in the outside fluid in two tubes. In one a bacillus was found, but no coccus, showing a contamination; and in the other tube there was a pure culture of staphylococcus. The outside broth of the remaining 10 sacs remained sterile, which was held clearly to establish the success of the method used in making the sacs without defects. Of the seven sacs inoculated with *B. typhosus* and incubated, all showed turbidity in the outside broth in less than 24 hours, with recovery of pure culture in each case.

The results of the experiments on the rabbits were as follows: One rabbit died from accidental injury. Three were anesthetized and as much peritoneal fluid removed, under aseptic precautions, as possible.

RABBIT 1.—Two to three c.c. of peritoneal fluid was removed 17½ hours after placement of the celloidin sac. Examination of a hanging-drop showed one to three motile organisms to a fluid. The peritoneal fluid was run into broth, well shaken, and plated on Conradi-Drigalski medium. Typical colonies were picked and identified, by cultural tests and agglutination reactions, to be *B. typhosus*.

<sup>1</sup> *Jour. of Exper. Med.*, 1901, 5, p. 635.



RABBIT 4.—*B. typhosus* recovered from peritoneal fluid 70 hours after placement of sac.

RABBIT 5.—*B. typhosus* recovered from peritoneal fluid 23 hours after placement of sac.

RABBIT 2.—This animal was bled at intervals and the serum tested for agglutinins by the microscopic tests. On the third day agglutination took place in a dilution 1:100, the fifth day 1:1,000, the seventh day 1:5,000, the 10th day 1:1,000, the 23d day 1:500.

The recovery of the typhoid bacillus from the peritoneal fluid of the rabbit not only adds further to the demonstration of the power of the bacillus to penetrate the celloidin membrane, but calls attention to the importance of this factor in animal work with certain bacteria. The rapid rise of agglutinins, from the use of the inoculated celloidin sac, suggests a feasible method of immunizing an animal against *B. typhosus*.

#### CONCLUSIONS.

The celloidin membrane, contrary to the accepted view of today, is not impervious to the passage of all bacteria.

The group of intestinal bacteria, and also *B. prodigiosus* and *B. pyocyaneus*, readily pass through celloidin sacs either by growth or direct passage.

The cocci—pneumococcus, staphylococcus, streptococcus—diphtheria and anthrax groups, and the spirilla—cholerae and metchnikovi—do not pass through celloidin membrane.

The bacterial permeability of the parchment sac is undoubtedly the same as that of the celloidin sac.

The results of experiments relative to the longevity of the typhoid bacillus, as determined by the use of the celloidin or parchment sacs, require retesting because of the partial escape of the bacilli from the sac.

In animal experiments with *B. typhosus*, when introduced in celloidin sacs into the peritoneal cavity, the factor of passage of the bacilli through the celloidin membrane must be considered in interpreting results. This is very probably true for all members of the intestinal group.

I wish to take this opportunity of thanking Professor E. O. Jordan for direction and suggestion during the course of this work.

## ROCKY MOUNTAIN SPOTTED FEVER IN THE RABBIT.\*

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THE first inoculation of rabbits made with the so-called "Rocky Mountain Spotted Fever" is mentioned by Wilson and Chowning<sup>1</sup> in their paper on *Piroplasmosis hominis*. One of their animals which was inoculated with 20 c.c. of blood from a human case died after eight days, showing on autopsy hemorrhagic areas under the skin, enlargement of the spleen, and hemorrhage into the kidney.

Ricketts,<sup>2</sup> in 1906, did not get positive results by the inoculation of rabbits with 1 c.c. and 4 c.c. respectively of defibrinated blood from two human patients. In one rabbit, however, he noticed a rise of temperature for about two or three days, four or five days after inoculation.

In a recent paper, Ricketts and Gomez<sup>3</sup> stated that the rabbit had been found susceptible to the virus as cultivated for a long time in the guinea-pig. The successive passage of the virus through the guinea-pig may have modified it in such a way that it became more virulent to the rabbit.

It is the purpose of this paper to describe features of the disease in rabbits as observed in recent experiments undertaken at the suggestion of Professor H. T. Ricketts.

### CLINICAL AND PATHOLOGICAL PHENOMENA.

After the inoculation of 0.5 to 1 c.c. of virus (defibrinated blood from infected guinea-pigs, drawn on the third or fourth day of fever) there occurs an incubation period of three to six days, marked by a slight rise of temperature which sometimes reaches 104° F. for one or two days, and then subsides before the fever of general invasion sets in. The latter is marked by a sudden rise in temperature to 104°-105° F., rarely higher, which persists for three to five days, after which it falls usually by lysis.

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<sup>1</sup> *Jour. Infect. Dis.*, 1904, 1, p. 48.

<sup>3</sup> *Jour. Infect. Dis.*, 1908, 5, p. 221.

<sup>2</sup> *Jour. Amer. Med. Assoc.*, 47, p. 36.

The rabbit, during the course of the disease, so far as weight and general health are concerned, is not profoundly affected, as a rule. The scrotum often becomes congested. One rabbit which died after withdrawal of blood from the heart showed petechial hemorrhages in the scrotum.

At autopsy the only gross changes found are the enlargement of the spleen and to a slight extent of the lymph glands. The spleen is also darker in color and softer in consistency than the normal organ. Other viscera do not show changes detectable to the naked eye.

The blood of the rabbit during the height of the disease is infective for the guinea-pig. For example, a rabbit which was inoculated intraperitoneally with 5 c.c. of virus had the following temperatures on successive days: 103.4, 103, 103.3, 102.2, 105.8, 104.9, 105.6, 105.7. Death followed from hemopericardium as a result of bleeding from the heart.

On the first day of fever, five days after inoculation, blood was drawn from the heart and injected into Guinea-pig 1,010 which ran the following course of fever on successive days: 102.8, 102.6, 103.1, 102.7, 105.6, 105.9, 105.8, 105.7, 105.8, 104.8, 104.8, 103, 103.6.

At autopsy the scrotum was hemorrhagic and gangrenous, and the ears gangrenous.

The blood of Rabbit 12, which showed a low degree of fever, was infective 12 days after inoculation. The following course of fever was shown by the rabbit on successive days: 103.3, 103, 103, —, 104, 104.2, 105, 104.8, —, 104.4, 103.6, 103.2, recovery.

Guinea-pig 1,176, which received 2 c.c. of fresh undefibrinated blood from Rabbit 12 on the 12th day after inoculation, had fever as follows: 104, 104.2, 104.6, —, 104.8, 104, 105.4, 105.6, 104, 101.4, 98.8, death.

At autopsy the spleen and lymph glands were found to be enormously enlarged, the adrenals showed punctiform hemorrhages, and the scrotum was congested and gangrenous.

Out of about 18 rabbits inoculated with the virus of spotted fever only one death could be ascribed to the disease, and this occurred 17 days after inoculation. After recovery many of the rabbits died from adventitious infections.

It has been possible to transmit the disease from one rabbit to

another six times by inoculating blood from the infected animal at the second to fourth day of high fever. Unfortunately the seventh rabbit that was inoculated died from an adventitious infection during the incubation period so that no conclusion can be reached as to how far the disease may be transmitted from one rabbit to another taking the temperature as the index of the disease.

## IMMUNITY IN THE RABBIT.

The blood of animals that have recovered from the disease contains protective antibodies in contrast to the blood of the normal rabbit. It has been found that 0.05 c.c. of defibrinated blood from the rabbit which has recovered affords a slight protection; 0.1 c.c. moderate and 0.3 c.c. complete protection against 1 c.c. of infected blood from the guinea-pig. This is shown in Tables 1 and 2.

The experiment illustrated in Table 1 shows that normal rabbit blood has little or no protective power for the guinea-pig.

TABLE 1.

## PROTECTIVE POWER OF NORMAL RABBIT BLOOD.

Virus from Guinea-pig 13 R, 3d day of fever: 1 c.c.\*

Normal rabbit blood in varying amounts.

Virus and blood mixed before injection, and the residue washed with salt solution and injected intraperitoneally

Test animals: guinea-pigs.

DATE 1908	GUINEA-PIG 21 R; NOR- MAL RABBIT BLOOD, 0.3 C.C.		GUINEA-PIG 22 R; NOR- MAL RABBIT BLOOD, 1 C.C.		GUINEA-PIG 20 R; CON- TROL; VIRUS ALONE; 1 C.C.	
	Temp.	Remarks	Temp.	Remarks	Temp.	Remarks
April 20 .....	.....		.....		.....	
" 21 .....	103.6		104.2		103.0	
" 22 .....	103.0		102.8		103.2	
" 23 .....	.....		.....		.....	
" 24 .....	105.4	Severe course of fever; gangrene of ears; no changes in genitalia; re- covery	106.2		105.6	
" 25 .....	.....		.....		.....	
" 26 .....	105.6		106.0	Autopsy typical for spotted fever	105.8	Autopsy typical for early spotted fever
" 27 .....	106.0		106.3		Killed	
" 28 .....	105.4		105.2			
" 29 .....	.....		.....			
" 30 .....	105.0		102.0			
May 1 .....	104.0		Died			
" 2 .....	103.8					
" 3 .....	.....					
" 4 .....	102.8					

\* Representing approximately 100 pathogenic doses.

TABLE 2.

PROTECTIVE POWER OF THE BLOOD OF "RECOVERED" RABBITS.

Virus from 56th "Eddy" passage: 1 c.c.\*

Immune defibrinated blood from "recovered" rabbits: varying amounts.

Immune blood and virus mixed before injection, and the residue washed with salt solution and injected intraperitoneally

Test animals: guinea-pigs.

DATE 1908	GUINEA-PIG 11 R; IMMUNE BLOOD, 0.5 C.C., FROM RAB- BIT 21, 8 DAYS AFTER SUBSIDENCE OF FEVER		GUINEA-PIG 8 R; IMMUNE BLOOD, 0.1 C.C., FROM RAB- BIT 21, 26 DAYS AFTER SUBSIDENCE OF HIGH FEVER		GUINEA-PIG 9 R; IMMUNE BLOOD, 0.3 C.C., FROM RAB- BIT 21		CONTROL; VIRUS ALONE, 0.01 C.C. GUINEA-PIG 16 R	
	Temp.	Remarks	Temp.	Remarks	Temp.	Remarks	Temp.	Remarks
April 3	.....		.....		.....		.....	
" 4	.....		.....		.....		.....	
" 5	103.0		102.0		103.4		103.6	
" 6	104.2		103.2		104.8		104.4	Severe course
" 7	.....		.....		.....	No distinct	.....	of fever; re-
" 8	104.8		103.2		103.6	course of	103.8	covery;
" 9	.....	Mild course	.....	Short course	.....	fever; irreg-	.....	scrotum and
" 10	104.2	of spotted	103.4	of mild fever;	103.2	ular rises	104.5	ears gan-
" 11	104.4	fever; scro-	103.6	recovery; no	102.6	probably ac-	106.6	grenous;
" 12	.....	tum swollen;	.....	changes in	.....	cidental;	.....	immunity
" 13	104.8	recovery	104.0	genitalia	102.6	recovery; no	107.2	test, May 26;
" 14	105.0		104.3		103.2	changes in	104.8	no fever
" 15	105.0		105.4		102.7	genitalia	106.2	developed
" 16	104.2		104.0		103.0		103.4	
" 17	.....		.....		.....		.....	
" 18	.....		.....		.....		.....	
" 19	103.4		103.5		103.4		103.5	
" 20	102.0		102.6		104.2		103.2	

\* Representing approximately 100 pathogenic doses

## SUMMARY.

The rabbit is susceptible to the virus of Rocky Mountain spotted fever as cultivated for a long time in the guinea-pig. The disease is milder in its symptoms than that produced in the monkey and guinea-pig. The blood of rabbits at the height of the disease is infective for guinea-pigs. It has been possible to transmit the disease from one rabbit to another through six animals successively. The blood of rabbits which have recovered contains bodies which protect against the disease, such bodies being largely or entirely absent from the blood of normal rabbits.

## THE INFLUENCE OF THE INGESTION OF DEAD TUBERCLE BACILLI UPON INFECTION.\*†

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DEAD tubercle bacilli, when injected into a guinea-pig or other animal, are capable of producing either local or general effects, depending chiefly on the number introduced. As to whether the ingestion of dead tubercle bacilli is harmful or beneficial, or without effect, the evidence is not clear, as there has been but little experimental work done on this subject.

In Germany and other European countries meat of tuberculous animals is not destroyed, as is required by the federal regulations of this country. Such meat in certain European countries is cooked under official surveillance and sold at a lower price. This is the so-called "Freibank system."

The question assumes great practical importance on account of the use of pasteurized milk, cooked meat from tuberculous animals, and other food products containing dead tubercle bacilli. It is of the utmost importance to determine if the susceptibility to tuberculosis is increased, decreased, or not altered by the previous eating of food containing tubercle bacilli killed by heat. The experiments which we wish to report were designed to simulate as closely as possible the effect produced by the ingestion of pasteurized milk or milk products.

The tubercle bacilli were heated at 60° C. for 30 minutes, mixed with butter, and fed to guinea-pigs daily (excepting Sundays) for 60 days. A low degree of heat was used to prevent altering the bacilli as much as possible and closely to simulate pasteurization. The animals were always given the dead bacilli mixed in the butter before receiving their usual morning feed. Control inoculations of the

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heated bacilli, made into the peritoneal cavity of five guinea-pigs, showed that the tubercle bacilli were dead.

In all these experiments the tubercle bacilli were fed as follows: The animal's mouth was opened and a little pellet of butter containing the tubercle bacilli placed upon the tongue. The animal was then released and it at once swallowed the material thus placed in the mouth. In placing the material upon the tongue it is convenient to use a piece of glass tubing into which the butter is pressed and can be squeezed out with a glass rod acting as a piston at the desired moment. In this manner animals may be fed without danger of inflicting wounds.

The butter used in these experiments was made in the laboratory from a specially good cream. The cream was obtained from a herd of tuberculin-tested cows, but was nevertheless first pasteurized to insure its freedom from live tubercle bacilli.

The culture used in these experiments is known as "Bovine Culture H," and was obtained from Dr. Leonard Pearson. It was isolated July 5, 1889, from the omentum of a guinea-pig which had been inoculated with an emulsion of caseous material from the mesenteric gland of a Jersey cow seven years old. The culture was grown on blood serum for 22 generations, then transplanted on glycerin-agar; it was either the 33d or 34th generation on glycerin-agar, and was virulent for guinea-pigs, rabbits, and cattle.

A four-weeks-old glycerin-potato culture was scraped off, suspended in water, and heated at 60° C. for 30 minutes; the bacilli were allowed to sediment and the supernatant water removed. The mass of dead tubercle bacilli was mixed with the butter in about equal proportions and kept in the ice-chest. At the time of feeding this was largely diluted with sterile butter and each guinea-pig given about 0.1 gm. of the mixture. Microscopic smears showed tubercle bacilli in each field lying singly or in groups.

The live bacilli used to infect the animals were taken from a 43-days-old glycerin-broth culture grown in six Fernbach flasks. The bacilli were dried at 37° C. and 100 gm. of the dried tubercle bacilli were mixed with an equal volume of butter. Each guinea-pig received about 0.1 gm. of the mixture, which was fed by the same method as described for the dead bacilli.

The details of the results are seen in the tables.

TABLE 1.


DEAD TUBERCLE BACILLI FED DAILY EXCEPT SUNDAYS FOR 60 DAYS PRIOR TO ONE FEEDING OF LIVE TUBERCLE BACILLI.

No. of Guinea-Pig	Day of Death	Initial Weight in Grams	Result	TUBERCULOSIS IN GLANDS										Remarks	
				Submental	Submaxillary	Cervical	Axillary	Inguinal	Bronchial	Anterior mediastinal	Retro-peritoneal	Sublumbar	Retro-hepatic		Mesenteric
1	80	300	Generalized tuberculosis	+	+	+	+	+	+	+	o	+	+	+	All had tuberculosis of liver, lungs, and spleen
2	92	275	"	+	+	+	+	+	+	+	+	+	+	+	
3	110	275	"	+	+	+	+	+	+	+	+	+	+	+	
4	112	260	"	+	+	+	+	+	+	+	+	+	+	+	
5	118	300	"	+	+	+	+	+	+	+	+	+	+	+	
6	129	270	"	+	+	+	+	+	+	+	+	+	+	+	
7	138	330	"	+	+	+	+	+	+	+	+	+	+	+	
8	142	340	"	+	+	+	+	+	+	+	+	+	+	+	
9	148	275	"	+	+	+	+	+	+	+	+	+	+	+	
10	155	260	"	+	+	+	+	+	+	+	+	+	+	+	
11	70	250	"	+	+	+	+	+	+	+	+	+	+	+	
12	71	320	"	+	+	+	+	+	+	+	+	+	+	+	
13	147	270	"	+	+	+	+	+	+	+	+	+	+	+	
14	112	300	"	+	+	+	+	+	+	+	+	+	+	+	
15	93	370	"	+	+	+	+	+	+	+	+	+	+	+	
16	71	290	"	+	+	+	+	+	+	+	+	+	+	+	
17	71	275	"	+	+	+	+	+	+	+	+	+	+	+	
18	71	245	"	+	+	+	+	+	+	+	+	+	+	+	
19	70	275	"	+	+	+	+	+	+	+	+	+	+	+	
20	70	290	"	+	+	+	+	+	+	+	+	+	+	+	
21	70	310	"	+	+	+	+	+	+	+	+	+	+	+	
22	70	310	"	+	+	+	+	+	+	+	+	+	+	+	
23	77	290	"	+	+	+	+	+	+	+	+	+	+	+	
24	98	330	"	+	+	+	+	+	+	+	+	+	+	+	
25	105	290	"	+	+	+	+	+	+	+	+	+	+	+	
26	92	260	"	+	+	+	+	+	+	+	+	+	+	+	
27	107	290	"	+	+	+	+	+	+	+	+	+	+	+	
28	111	260	"	+	+	+	+	+	+	+	+	+	+	+	
29	113	275	"	+	+	+	+	+	+	+	+	+	+	+	
30	117	240	"	+	+	+	+	+	+	+	+	+	+	+	
31	117	275	"	+	+	+	+	+	+	+	+	+	+	+	
32	176	275	"	+	+	+	+	+	+	+	+	+	+	+	
33	119	275	"	+	+	+	+	+	+	+	+	+	+	+	
34	151	290	"	+	+	+	+	+	+	+	+	+	+	+	
35	151	285	"	+	+	+	+	+	+	+	+	+	+	+	
36	151	300	"	+	+	+	+	+	+	+	+	+	+	+	
37	127	275	"	+	+	+	+	+	+	+	+	+	+	+	
38	185	355	"	+	+	+	+	+	+	+	+	+	+	+	
39	151	315	"	+	+	+	+	+	+	+	+	+	+	+	
40	130	270	"	+	+	+	+	+	+	+	+	+	+	+	
41	120	285	"	+	+	+	+	+	+	+	+	+	+	+	
42	126	300	"	+	+	+	+	+	+	+	+	+	+	+	
43	120	300	"	+	+	+	+	+	+	+	+	+	+	+	
44	120	260	"	+	+	+	+	+	+	+	+	+	+	+	
45	148	300	"	+	+	+	+	+	+	+	+	+	+	+	
46	140	275	"	+	+	+	+	+	+	+	+	+	+	+	
47	133	275	"	+	+	+	+	+	+	+	+	+	+	+	

Average 113.

+ = Tuberculosis; o = Normal; . = Not noted.

TABLE 2.  
CONTROLS : ONE FEEDING OF LIVE TUBERCLE BACILLI.

No. of Guinea-Pig	Day of Death	Initial Weight in Grams	Result	TUBERCULOSIS IN GLANDS										Remarks		
				Submental	Submaxillary	Cervical	Axillary	Inguinal	Bronchial	Anterior mediastinal	Retro-peritoneal	Sublumbar	Retro-hepatic		Mesenteric	
1	79	320	Generalized tuberculosis	o	o	o	o	+	+	o	o	+	+	+	All had tuberculosis of liver, lungs, and spleen	
2	79	320	"	+	+	+	o	+	+	+	+	+	+	+		
3	79	275	"	+	+	+	+	+	+	+	+	+	+	+		
4	79	275	"	+	+	+	+	+	+	+	+	+	+	+		
5	71	260	"	+	+	+	+	+	+	+	+	+	+	+		
6	105	275	"	+	+	+	+	+	+	+	+	+	+	+		
7	80	230	"	+	+	+	+	+	+	+	+	+	+	+		
8	105	265	"	+	+	+	+	+	+	+	+	+	+	+		
9	101	300	"	+	+	+	+	+	+	+	o	o	o	o		Lesions above diaphragm. Ax. and ing. glands slightly enlarged
10	94	300	"	+	+	+	o	o	+	+	+	+	+	+	+	
11	108	285	"	+	+	+	o	o	+	+	+	+	+	+	+	
12	107	210	"	+	+	+	+	+	+	+	+	+	+	+	+	
13	108	270	"	+	+	+	o	o	+	+	+	+	+	+	+	
14	111	280	"	+	+	+	o	o	+	+	+	+	+	+	+	
15	111	230	"	+	+	+	o	o	+	+	+	+	+	+	+	
16	111	335	"	+	+	+	o	o	+	+	+	+	+	+	+	
17	111	255	"	+	+	+	o	o	+	+	+	+	+	+	+	
18	114	315	"	+	+	+	+	+	+	+	+	+	+	+	+	
19	116	285	"	+	+	+	o	+	+	+	+	+	+	+	+	
20	118	350	"	+	+	+	o	+	+	+	+	+	+	+	+	
21	118	320	"	+	+	+	o	o	+	+	+	+	+	+	+	
22	118	335	"	+	+	+	o	o	+	+	+	+	+	+	+	Perit. surface of colon studded with tubercles  Chloroformed "
23	110	300	"	+	+	+	o	o	+	+	+	+	+	+	+	
24	118	275	"	+	+	+	o	o	+	+	+	+	+	+	+	
25	176	305	"	o	+	+	o	o	+	+	+	+	+	+	+	
26	176	250	Normal Generalized tuberculosis	+	+	+	o	o	+	+	+	+	+	+	+	
27	172	335	"	+	+	+	o	o	+	+	+	+	+	+	+	
28	157	300	"	+	+	+	o	o	+	+	+	+	+	+	+	
29	128	255	"	+	+	+	+	+	+	+	+	+	+	+	+	
30	128	310	"	+	+	+	+	+	+	+	+	+	+	+	+	
31	126	285	"	+	+	+	+	+	+	+	+	+	+	+	+	
32	122	205	"	+	+	+	+	+	+	+	+	+	+	+	+	
33	123	285	"	+	+	+	+	+	+	+	+	+	+	+	+	
34	122	235	"	+	+	+	+	+	+	+	+	+	+	+	+	
35	146	220	"	+	+	+	+	+	+	+	+	+	+	+	+	
36	142	270	"	+	+	+	+	+	+	+	+	+	+	+	+	
37	140	290	"	+	+	+	+	+	+	+	+	+	+	+	+	
38	120	270	"	+	+	+	+	+	+	+	+	+	+	+	+	All lesions practically above diaphragm  Killed by accident
39	132	255	"	+	+	+	o	+	+	+	+	+	+	+	+	
40	84	260	"	+	+	+	+	+	+	+	+	+	+	+	+	
41	85	275	"	+	+	+	+	+	+	+	+	+	+	+	+	
42	84	340	"	+	+	+	+	+	+	+	+	+	+	+	+	
43	158	325	"	+	+	+	+	+	+	+	+	+	+	+	+	
44	49	200	"	+	+	+	+	+	+	+	+	+	+	+	+	
45	63	310	"	+	+	+	+	+	+	+	+	+	+	+	+	
46	62	275	"	+	+	+	+	+	+	+	+	+	+	+	+	

Average 108.

+ = Tuberculosis; o = Normal; . = Not noted.

One hundred guinea pigs about six weeks old were used in these experiments. They were divided into two lots; the first lot, consisting of 50, was fed for 60 days on the heated tubercle bacilli; the second lot, also consisting of 50 guinea-pigs, which served as the controls, was kept under the same conditions, but did not receive the heated bacilli. The entire 100 at the end of 60 days were given one feeding of the 43-days-old live tubercle bacilli in butter. During the entire course of the experiments all the animals were kept under exactly similar conditions. As soon as an animal died it was carefully autopsied, especial note being made of the involvement of the lymphatic glands.

Under the conditions of the experiments it is evident that the feeding of the dead tubercle bacilli to the guinea-pigs did not alter their susceptibility. It seemed to have no evident effect upon the subsequent course of the disease. It is not safe to conclude from this that the ingestion of dead tubercle bacilli under all circumstances is a harmless procedure, although this is perhaps indicated.

As a result of this work several interesting observations were noted. One is, that none of the animals fed with the dead bacilli lost weight during the 60 days this process continued. It is also evident that if a guinea-pig be given a sufficient amount it may be infected by ingestion, for all the animals with one exception became infected. In one instance (Guinea-pig 9, Table 2) the lesions of ingestion tuberculosis were all above the diaphragm; this guinea-pig had three enlarged and caseous glands in the neck, the bronchial and mediastinal glands were tuberculous, and the lungs tuberculous; the liver, spleen, and other abdominal organs were normal.

It is also evident that it takes much longer for guinea-pigs to die of ingestion tuberculosis than when the same material is inoculated into the peritoneal cavity. Five controls, which had received the same material into the peritoneal cavity, died in 13, 15, 15, 17, and 37 days respectively, whereas the average from Table 1 was 113 days and from Table 2 it was 108 days.

# THE RELATIVE PROPORTION OF BACTERIA IN TOP MILK (CREAM LAYER) AND BOTTOM MILK (SKIM MILK), AND ITS BEARING ON INFANT FEEDING.\*†

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IN the course of a study on tubercle bacilli in market milk<sup>1</sup> and of a later study on the best procedure for their detection in milk it was noticed that when guinea-pigs were inoculated with the cream a very much higher percentage died from acute infections than when the sediment was used. The inference was natural that the cream contained more bacteria than the bottom milk or sediment. A few preliminary examinations having shown this supposition to be correct, a study was begun as to the number of bacteria in the whole milk, the bottom milk or sediment, and the cream, both that collected by gravity and by centrifugation.

The relative number of bacteria in the top milk and in the bottom milk is a subject of very great importance in the modification of milk for infant-feeding. All the writers on pediatrics and infant-feeding give formulae for the modification of milk based upon the use of various amounts of top milk. My studies show that top milk, such as is advised for use in the above formulae, contains from 10 to 500 times as many bacteria per c.c. as the mixed milk. This preponderance of bacteria in top milk may account for the fact that sometimes children do not thrive on modified milk when made from top milk, but improve when the whole milk is used for modification.

The various bacteria causing acute infections, as well as tubercle bacilli, are more numerous in the top milk than in the bottom milk. In many cases this difference is more than a hundred fold and, as infection must depend to some extent on the number of bacteria intro-

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<sup>1</sup> Anderson, John F., "The Frequency of Tubercle Bacilli in the Market Milk of the City of Washington, D. C." *Bull. No. 41, Hyg. Lab., U. S. Pub. Health and Mar. Hosp. Serv.*, 1908, p. 163.

duced into the body, too little attention has been given to the question of the number of bacteria in top milk when used for infant-feeding. Oftentimes when infants are taken off breast milk and put on modified cow's milk made from top milk it is found that, in spite of various modifications containing varying percentages of proteid, the milk fails to agree with the infant. In those cases which develop diarrhea the fault may not always be in the proteids, but in the large number of bacteria in the top milk used for the preparation of the formula.

The greater frequency of intestinal tuberculosis in young children may be due, not only to the fact that they use a large amount of milk, but because top milk, which contains more tubercle bacilli per c.c. than the whole milk, is used in the preparation of modified-milk formulae.

The literature upon the subject of the relative number of bacteria in top milk and in bottom milk is very slight. None of the writers seems to have realized the great importance of the subject in its relation to infant feeding.

Wyss<sup>1</sup> investigated the question whether, by centrifugation, milk is freed of a part of its bacteria or whether the organic substance of the milk sediment does not, for the greater part, consist of bacteria. His work resulted in the determination that the precipitate was seven times richer in bacteria than the centrifugalized milk.

Scheurlen<sup>2</sup> found in his experiments with pathogenic bacteria other than the tubercle bacilli in centrifugalized milk a marked bacterial increase in the sediment and the cream, and a marked decrease in the bottom milk. Among others, he gives the following example of the distribution of bacteria by centrifugalization of milk purchased from the Bolle dairy in Berlin:

Milk contained.....	2,050,000	bacteria per c.c.
Cream.....	8,500,000	" "
Bottom milk .....	700,000	" "
Milk dirt .....	18,000,000	" in 0.6 c.c.

Niederstadt<sup>3</sup> draws the conclusion that in the centrifugalization of milk 75 per cent of the bacteria go to the cream, while the rest remain in the bottom milk. Centrifugalization does not free the milk of its bacteria.

Wilkens<sup>4</sup> found that the greater part of the bacteria go into the cream on centrifugalizing and that the total number was reduced by the process, not alone through the removal of the sediment; he supposed that some of them were killed.

Russell<sup>5</sup> states that cream, whether secured by gravity or by a cream separator, is invariably richer in bacteria than the skimmed milk of the same age. A sample of

<sup>1</sup> *Centralbl. f. Bakt.*, 1880, 6, p. 587.

<sup>2</sup> *Arb. a. d. kais. Gesundheitsamte*, 1891, 7, pp. 269-82.

<sup>3</sup> *Kochs Jahresber. u. d. Fortschr. in der Lehre v. d. Gärungs-Organismen*, 1893, 4, p. 205.

<sup>4</sup> *Centralbl. f. Bakt., Ref.*, 1894, 16.

<sup>5</sup> *Outline of Dairy Bacteriology*, Madison, 1894, pp. 127.



milk might have less than 100,000 bacteria per c.c. in the skimmed part, while the number in the cream layer would be several millions. Gravity raised cream is usually richer in bacteria than separator cream, mainly because it is older. He cites Poff and Becker as saying that a sample of milk which, before it was separated, had 73,000 bacteria per c.c., had the following after separation:

Cream.....	58,275
Skimmed milk.....	21,500
Slime.....	43,900

Backhaus and Cronheim<sup>1</sup> did not find that the cream obtained by centrifugalization was much richer than the bottom milk in bacteria. They obtained the following results with two samples of milk.

Whole milk.....	{ 1,910,000 bacteria per c.c.		
	{ 3,420,000	"	"
Cream.....	{ 1,740,000	"	"
	{ 3,110,000	"	"
Bottom milk.....	{ 1,450,000	"	"
	{ 2,600,000	"	"

Severin<sup>2</sup> states that, under the influence of centrifugalization, the milk is separated into three layers: The dirt particles, being the heaviest of the ingredients, are thrown to the periphery where they remain clinging to the walls of the centrifuge; then comes the whey ring; while in the central portion of the centrifuge is the cream, which is the lightest part of the milk. The bacteria of the milk are also influenced by the centrifugalization. Organisms with higher specific gravity than the milk are thrown to the periphery; those with lower are removed to the center. This peculiar distribution of the bacteria in the milk is manifestly complicated by the question whether the organisms possess specific movement or not.

Hess<sup>3</sup> found, in his study on the distribution of bacteria in bottled milk, that the bacteria are much more numerous in the upper layers of the cream, gradually becoming fewer in the lower portion of the bottle. The upper two ounces contain the greatest number of bacteria. He found this to be true of the tubercle bacilli, as well as the staphylococci and other bacteria.

#### METHODS.

Pint bottles of milk were purchased from different dairies each day and at once brought to the laboratory. As soon as received the milk was packed in cracked ice in order to allow the cream to separate as well as possible; two hours was usually sufficient for this purpose, though it was found that some bottles failed to show a distinct cream-line in that time. An effort was made to avoid purchasing the milk from the same dairy more than twice.

The following dilutions were, with a few exceptions, used throughout the work: 1 c.c. of the milk + 99 c.c. sterile water; 0.1 c.c., which

<sup>1</sup> *Kochs Jahresber. u. d. Fortschr. in der Lehre v. d. Gärungs-Organismen*, 1897, 8, p. 150.

<sup>2</sup> *Centralbl. f. Bakt.*, Abt. 2, 1905, 14, p. 605.

<sup>3</sup> *Pediatrics*, New York, August, 1908.

represented a dilution of 1:1,000, was used for the first plate. One c.c. of the first dilution was added to 99 c.c. sterile water; 1 c.c. of this dilution represented 0.0001 c.c. of the original milk, and 0.1 c.c. represented 0.00001 c.c. Three plates were made from each sample. The mixtures were vigorously shaken in order to break up the clumps of bacteria and obtain as uniform a suspension of bacteria as possible. The amount of each dilution to be plated was measured directly into a petri dish and melted agar poured in the plate. The plates were kept at 37° C. for 24 hours and the colonies then counted.

THE RELATIVE NUMBER OF BACTERIA IN THE CREAM LAYER AND IN THE SEDIMENT LAYER OF BOTTLE MILK.

In the first series of comparative counts 35 samples of milk were studied. From each sample counts were made of the number of bacteria in the cream layer and in the sediment layer.

TABLE 1.  
NUMBER OF BACTERIA IN THE GRAVITY CREAM AND SEDIMENT LAYERS.

Sample	Bacteria in Cream Layer	Bacteria in Sediment Layer
1A.....	405,000	2,000
2A.....	4,500,000	83,000
2B.....	1,728,000	77,000
2C.....	16,200,000	1,674,000
3A*.....	114,000,000	106,800,000
3B.....	62,100,000	2,600,000
3C*.....	637,200,000	456,000,000
4A.....	307,200,000	12,000,000
4B.....	46,800,000	2,200,000
4C.....	194,400,000	22,200,000
5A.....	145,200,000	1,500,000
5B.....	306,000,000	1,640,000
5C.....	82,400,000	2,760,000
6A.....	70,800,000	9,300,000
6B.....	67,200,000	1,200,000
6C.....	16,680,000	1,055,000
7A*.....	300,000	200,000
7B.....	800,000	30,000
7C.....	6,200,000	200,000
8A.....	10,500,000	1,200,000
8B*.....	360,000	280,000
8C.....	7,200,000	260,000
8D.....	210,000	40,000
8E.....	140,000	10,000
8F.....	140,000	
8G.....	1,000,000	200,000
8H.....	80,000	20,000
8I*.....	120,000	100,000
8J.....	870,000	70,000
9A.....	1,800,000	312,000
9B.....	160,800,000	6,360,000
9C.....	7,080,000	800,000
10A.....	6,200,000	240,000
10B.....	248,400,000	11,200,000
10C.....	152,400,000	51,600,000
Average.....	69,211,000	4,360,000

\* Cream not separated after standing two hours; excluded from calculation of the averages.

For the cream counts the point of the pipette was put just below the surface and 1 c.c. drawn into it and used for the dilutions.

For the sediment-layer counts the pipette was plunged into the bottom of the bottle, 1 c.c. drawn up and then diluted.

It was noted that in those milks which did not show a good cream-line in two hours or which contained much dirt, the difference in the number of bacteria in the cream and sediment layers was not as marked as in those which had a distinct cream-line or were fairly free from dirt.

As will be seen from Table 1, the average number of bacteria in the cream layer of the 30 samples used in the calculation of the averages was 69,211,000. The average number in the sediment layer of the same sample was 4,360,000; or, there were about 16 times as many bacteria in the cream layer as in the sediment layer.

#### RELATIVE NUMBER OF BACTERIA IN GRAVITY CREAM, CENTRIFUGAL CREAM, AND IN THE SEDIMENT LAYER.

In studying the relative number of bacteria in gravity and centrifugally raised cream and in the sediment layers, the same sample of milk was used. After planting from the cream and sediment layers of the bottle of market milk, as in the preceding series, the milk was thoroughly mixed and 200 c.c. placed in a centrifuge flask. It was centrifugalized one hour at about 1,500 revolutions per minute. The cream was carefully removed and 1 c.c. of it was diluted and plates made in the usual way. After the cream had been removed 1 c.c. of the sediment was diluted and plated.

It will be seen from Table 2 that the average number of bacteria in the 26 samples of gravity cream was 68,690,000 and in the sediment 4,840,000; that is, about 14 times as many bacteria were in the cream as in the sediment.

When we come to compare the number of bacteria in the centrifugalized cream and sediment we find that the average in the cream was 97,690,000, and in the sediment 18,840,000; or only about 5.1 times as many in the cream as in the sediment.

The average number of bacteria in the mixed, or whole, milk was 14,388,000; which is slightly less than the average number in the centrifugalized sediment of the same milk.

TABLE 2.

RELATIVE NUMBER OF BACTERIA IN GRAVITY CREAM, CENTRIFUGAL CREAM, AND IN THE SEDIMENT LAYER.

SAMPLE	GRAVITY		CENTRIFUGALIZED		WHOLE MILK
	Cream Layer	Sediment Layer	Cream Layer	Sediment Layer	
11A*	2,800,000	35,000	2,200,000	1,100,000	300,000
11B*	20,000,000	28,000	9,500,000	640,000	150,000
11C*	40,200,000	5,700,000	56,400,000	4,860,000	7,400,000
12A*	72,000,000	10,800,000	106,000,000	8,500,000	16,500,000
12B*	88,800,000	1,040,000	17,100,000	1,000,000	9,360,000
12C*	702,000,000	50,400,000	248,400,000	151,200,000	134,400,000
13A*	3,700,000	230,000	17,500,000	756,000	700,000
13B*	93,400,000	190,000	167,400,000	11,000,000	17,100,000
13C*	50,400,000	630,000	70,600,000	3,320,000	4,800,000
14A*	73,800,000	4,800,000	141,600,000	5,550,000	12,600,000
14B*	58,800,000	4,100,000	126,000,000	21,000,000	8,600,000
14C*	30,380,000	3,100,000	60,000,000	10,600,000	8,000,000
15A†	11,400,000	570,000	35,400,000	2,300,000	2,700,000
15B†	25,400,000	2,300,000	36,600,000	13,800,000	4,600,000
15C†	297,000,000	13,100,000	520,200,000	60,000,000	46,800,000
16A†	82,800,000	1,430,000	480,600,000	9,000,000	38,400,000
16B†	1,200,000	150,000	4,700,000	400,000	380,000
16C†	54,240,000	20,200,000	263,100,000	162,000,000	46,500,000
17A†	400,000	42,000	1,700,000	1,000,000	210,000
17B†	23,200,000	2,500,000	64,800,000	3,200,000	4,100,000
17C†	25,800,000	920,000	22,000,000	432,000	3,600,000
17D†	10,600,000	3,800,000	64,800,000	6,400,000	6,800,000
18A‡	440,000	100,000	650,000	220,000	50,000
18B‡	210,000	5,000	250,000	60,000	47,000
18C‡	350,000	13,000	770,000	71,000	35,000
18D‡	406,000	22,000	620,000	45,000	46,500
Average.....	68,690,000	4,840,000	97,690,000	18,840,000	14,388,000

\* = Sediment not noted; † = Dirty sediment; ‡ = No visible sediment.

The enormous difference sometimes found in the bacterial content of the cream and sediment is shown in Sample 13B, in which the cream contained 486 times as many bacteria as the sediment.

From this series it is plain that centrifugally raised cream has slightly more bacteria than gravity raised cream, and centrifugal sediment about four times as many as gravity sediment. This seems not to be dependent upon the presence of dirt, as is seen from the last four samples which were free from visible sediment.

#### THE RELATIVE NUMBER OF BACTERIA IN GRAVITY CREAM, SKIM MILK, SEDIMENT, AND THE WHOLE MILK.

In studying the relative number of bacteria in gravity raised cream, sediment, skim milk, and the whole milk six samples of milk were examined. The milk was bought in the open market in pint or quart bottles and examined soon after reaching the laboratory. All showed a distinct cream-line. After planting from the cream and sediment layers, 1 c.c. was taken from the mid portion some distance below

the cream-line and diluted. After these samples were taken the bottle of milk was well shaken and the sample of whole milk taken, diluted, and plated.

It will be seen from Table 3 that the average number of bacteria in the whole was 2,708,000, in the cream 15,416,000, in the sediment

TABLE 3.  
THE RELATIVE NUMBER OF BACTERIA IN GRAVITY CREAM, SKIM MILK, SEDIMENT, AND IN THE WHOLE MILK.

Sample	Whole Milk	Cream	Skim Milk	Sediment
19A.....	4,500,000	42,800,000	1,685,000	2,000,000
20.....	9,10,000	7,400,000	665,000	570,000
21.....	7,700,000	18,800,000	7,200,000	4,200,000
22.....	870,000	6,700,000	580,000	240,000
23.....	510,000	2,100,000	255,000	220,000
24.....	2,000,000	14,700,000	1,920,000	1,200,000
Average.....	2,708,000	15,416,000	2,050,000	1,405,000

layer 1,405,000, and in the skim milk 2,050,000. From this it will be seen that there is little difference in the number of bacteria in the whole milk, sediment layer, and skim milk, while the cream layer contained about eight times as many per c.c.

THE RELATIVE NUMBER OF BACTERIA IN CENTRIFUGAL CREAM,  
THE SKIM MILK, SEDIMENT, AND IN WHOLE MILK.

In studying the relative number of bacteria in the cream, sediment, and skim milk of centrifugalized milk seven samples of market milk were examined. The bottle of milk was thoroughly shaken and, after plates had been made of the whole milk, 200 c.c. of the mixed milk were placed in a centrifuge flask and centrifugalized for one hour. The cream was carefully removed, diluted, and plated. Plates were then made from the sediment layer and the skim milk.

It will be seen from Table 4 that the average number of bacteria in the whole milk was 619,000, in the cream 4,500,000, in the sediment 725,000, and in the skim milk only 119,700 per c.c. There was about the same number of bacteria in the whole milk and in the sediment, while the cream contained about 37 times as many bacteria as the skim milk. The great mass of bacteria under the influence of centrifugalization either went up in the cream or down with the sediment, leaving the skim milk with comparatively few bacteria.

TABLE 4.  
THE RELATIVE NUMBER OF BACTERIA IN CENTRIFUGAL CREAM, SKIM MILK, SEDIMENT,  
AND IN WHOLE MILK.

Sample No.	Cream	Skimmed Milk	Sediment	Whole Milk
25.....	2,000,000	44,666	90,000	120,000
26.....	1,300,000	44,300	52,500	100,000
27.....	600,000	6,500	60,000	120,000
28.....	18,000,000	210,000	800,000	2,400,000
29.....	7,800,000	470,000	3,530,000	790,000
30.....	750,000	40,000	400,000	323,000
31.....	1,050,000	22,000	140,000	480,000
Average.....	4,500,000	110,700	725,000	619,000

## SUMMARY.

The relative number of bacteria in top milk and bottom milk is of great importance in the modification of milk for infant feeding. Top milk sometimes contains from 10 to 500 times as many bacteria per c.c. as the mixed milk.

The preponderance of bacteria in top milk may explain why infants sometimes do not thrive on modified milk made from top milk.

As infection depends to some extent on the number of organisms introduced into the body, top milk on account of its higher bacterial content may sometimes be more harmful than skim milk.

In 30 samples of bottle milk examined the average number of bacteria in gravity raised cream was 69,211,000 and in the sediment layer 4,360,000 bacteria per c.c.

In 26 samples of milk the average number of bacteria in gravity and centrifugally raised cream, in the sediment layer, and in the mixed milk was:

GRAVITY		CENTRIFUGALIZED		WHOLE MILK
Cream Layer	Sediment Layer	Cream Layer	Sediment Layer	
68,690,000	4,840,000	96,690,000	18,840,000	14,388,000

In six samples of milk the average relative number of bacteria in the gravity cream was 15,416,000, in the skim milk 2,050,000, in the sediment layer 1,405,000, and in the whole milk 2,708,000.

In seven samples of milk the average relative number of bacteria in the centrifugally raised cream was 4,500,000, in the sediment layer 725,000, in the skim milk, 119,700, and in the whole milk 619,000.



One sample of milk contained 500 times as many bacteria per c.c. in the cream as in the bottom milk.

When milk is centrifugalized the great mass of bacteria go up with the cream; a lesser number is carried down in the sediment. The skim milk contains many times less numbers of bacteria per c.c. than the cream or sediment layers.

Centrifugally raised cream contains more bacteria per c.c. than the gravity raised cream from the same milk.





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## MILKSICKNESS.\*†

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† This disease has been commonly known under the following names: Milksickness, Sick stomach, Swamp sickness, Tires, Trembles, Slows (sometimes corrupted to Sloes), Stiff joints, Puking fever, River sickness, and Alkali poisoning. More or less fanciful and pedantic designations have also been applied to it, such as Caconemia, Colica tremmentia, Paralysis intestinalis, Morbo lacteo, Ergodeleteria, Mukosma, Syro, and Lacemesia; none of these latter titles appears to have come into general use.

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## SUMMARY AND CONCLUSIONS

THE first printed account of milksickness seems to have been from the pen of Dr. Daniel Drake, "the Franklin of Cincinnati," who published in 1810 a brief note on a "New Disease" in his *Notices Concerning Cincinnati*. This publication is now not easy of access, but the note on milksickness was reprinted by Drake in the form of an editorial addition to an article by McCall (1830). From its historical interest the note is here reproduced.

"NEW DISEASE. In the spring of 1809 Dr. Barbee, of Virginia, on returning from a visit to the Madriver County in this state, gave me some information concerning a new and formidable disease which had appeared among the settlers of that tract. Since that time I have been able to collect several additional facts respecting it from different persons, more especially Mr. Wm. Snodgrass and Mr. John M'Kag, two intelligent and respectable inhabitants of that country, who have several times experienced the disease in their persons or families. A summary of the whole is here given that physicians may determine how far it deserves the appellation of a new disease.

It almost invariably commences with general weakness and lassitude which increases in the most gradual manner; about the same time, or soon after, a dull pain, or rather soreness, begins to affect the calves of the legs, occasionally extending up to the thighs. The appetite becomes rather impaired, and in some cases nearly suspended, sensations of a disagreeable kind affecting the stomach; upon taking a little food, a greater disposition for it is generated, and more agreeable feelings are introduced throughout the whole system. Intestinal constipation in this, as in subsequent periods of the disease, exists in a very high degree. A strong propensity to sleep occurs, and according to Dr. Barbee the pulse is "full, frequent, round, and somewhat tense, but regular." During this stage exercise of any kind is highly detrimental, and if persisted in soon induces loathing and nausea at the stomach. If the patient repose upon first experiencing the symptoms, they generally cease and he is allowed a longer exemption from the vomiting that awaits him. Sooner or later, however, that symptom almost invariably succeeds the predisposition we have described, and either proves fatal in

one, two, three, or more days, or leaves the patient in a most exhausted state from which he recovers, only to sustain, at no distant period, a repetition of the same attack.

The matter ejected is somewhat bilious, but much oftener sour, and so acrid that its action on the throat, in one case (which proved fatal), was likened to that of boiling water. Towards the close of mortal cases, it is occasionally very dark colored, so that it has been compared to that very convenient and fashionable object of similitude, coffee grounds. At this time the intestinal constipation is very great. Mr. Snodgrass knew one patient in whom it continued for nine days, throughout which he took no food whatever, and vomited during six of them. After such an attack the propensity of it is destroyed, and an uncommon degree of watchfulness is produced. The patient remains languid, and his face and person generally become rather tumid. His skin is cool, palish, and frequently affected with clamminess. He has a disagreeable burning sensation at his stomach, and hot eructations are very troublesome. The thirst is considerable. The breath is peculiarly disgusting, even loathsome. The appetite is generally poor, and the inclination to costiveness remains. These symptoms often continue for several months, during which the patient experiences frequent returns of the vomiting. But at length, more especially upon the approach of winter, they gradually wear away, leaving the patient considerably worse than they found him, and liable to a fresh attack the ensuing summer.

Nothing like *regular* periodical exacerbations is observable in this disease, no chilliness occurs, the color of the skin and eyes does not deviate widely from that of health, and gives no striking indication of bile, there is no pain in the region of the liver, nor in the shoulder, it does not terminate in dropsy, nor are there any symptoms which bespeak it a disguised or anomalous intermittent. It however prevails (though not exclusively) in aguish situations, and intermitting diseases are thought to have declined since its appearance.

It affects all ages and conditions, and both sexes, indiscriminately, except probably very young children. They, however, are not wholly exempt from it. Emigrants are not peculiarly liable to it. It was first observed in the summer of 1806, and it is thought annually to extend its geographical range and to become more intense. It sometimes commences in July or before, but oftener in August and continues till the approach of winter, when it generally but not always subsides.

The cure of this disease seems hitherto to have been left chiefly to the people, who have not yet discovered any certain method. Purging was a remedy that naturally suggested itself, and by some it has been thought very serviceable, more especially when effected by aloes; but others assert that they have frequently known a cathartic to increase the vomiting, and therefore rely more on enemata. All agree, however, that intestinal obstructions are to be overcome; and that the less the means made use of affect the stomach, the better. Vomits evidently do harm. Blisters to the gastric region are considered the most efficient remedy. Tonics have been used but no great benefit appeared to arise from them. Wine and salted meats, however, have appeared to do good, and are relished beyond anything else. Indeed, eating a little frequently, whether an inclination exist or not, has been found a good palliative. It relieves the stomach from the gnawing which so perpetually exists. Alkaline lye has been used in one case, it gave some temporary relief, but not more than almost any other substance which might be received into the stomach. Bleeding has occasionally been resorted to, but with doubtful advantage. Ardent spirits appears to render the disease worse; it is not, however, much sought after, all inclination for it generally being destroyed.



Tea and coffee, also, with several other articles of diet, which were agreeable before the disease, are in many cases disliked for a long time after.

The disease is unequivocally observed to affect four domestic animals: the horse, the cow, the sheep, and the dog. It is often fatal to the two former, but not so fatal to the latter. It as frequently attacks horses in the winter as summer, and sometimes kills them in 24 hours.

It prevails chiefly in the neighborhood of Staunton on the Great Miami and in the county south of Madriver, between Dayton and Springfield. In these tracts ponds and marshes occasionally occur, more especially in the former. The soil and water are calcareous. The timber generally oak."

The disease seems to have existed in parts of the United States for some time before the date of Drake's note. Coleman (1822), writing of "the disease generally known by the name of sick-stomach," states that it "has been prevalent in some of the western counties of the state of Ohio, since their first settlement," and Drake (1836) gives currency to the report "that this malady was observed in some parts of North Carolina more than sixty years ago [i. e., before 1776], and that it has been an endemic in certain localities of Tennessee, Kentucky, and Ohio from their first settlement." Drake's correspondent cited in this report gives the names of two persons who died of the disease in Kentucky about 1795. McCall (1823) specifically mentions several cases occurring in parts of North Carolina between 1779 and 1800.

Lea (1821), writing of the disease in the state of Tennessee, makes the interesting statement that "as soon as settlements commenced in the county of Franklin, about 12 or 15 years since, near the mountain, many cattle were lost from some unknown poison, the nature of which is still a mystery among the inhabitants. Occasionally, whole herds were found dead in some sequestered cove of the mountains." Carney (1847) speaks of the disease being noticed, "I think sixty or seventy years ago in the Carolinas," and continues: "Upwards of fifty years since, it prevailed in Kentucky." Rawlings (1874) refers to "its first avowed recognition in the State of North Carolina—now over ninety-six years ago" (i. e., prior to 1778). It thus seems fairly clear that the disease was known in the Carolinas at least as early as 1780 and probably before that time. It appeared somewhat later in parts of Kentucky and Tennessee and a little later still in Ohio, Illinois, and Indiana. According to Rawlings (1874), Wood stated that "the disease followed emigration in its westward march from North Caro-

lina to Tennessee and Kentucky, and prevailed in various parts of Ohio, Indiana, and Illinois." It is on record that the mother of Abraham Lincoln died of milksickness at Pigeon Creek in southern Indiana in 1818 (Nicolay and Hay, 1890).

Some writers assert that mention of this disease occurs in Father Hennepin's account of his travels, but we have not been able to find anything in Hennepin's writings that could bear such an interpretation. Dr. G. R. Thwaites who is probably the historian most familiar with Hennepin's writings informs us that he does not know of any reference that Hennepin makes to this subject.

The disease was brought more than once to the attention of early travelers in the Middle West. Faux (1823) reports its presence in Old Vincennes, Ind., in 1819, and its presence in northwestern Missouri was noted by Long's Expedition about the same time (Long, 1823). M'Kenney (1846) met with milksickness in 1827 along the Mississippi River about 18 miles above St. Louis, and Flagg (1836) gives the following historical note based on information received by him while traveling in Monroe County, Ill., in 1836-37: "A mysterious disease called the '*milk* sickness'—because it was supposed to be communicated by that liquid—was once alarmingly prevalent in certain isolated districts of Illinois. Whole villages were depopulated; and though the mystery was often and thoroughly investigated the cause of the disease was never discovered. By some it was ascribed to the milk or to the flesh of cows feeding upon a certain unknown poisonous plant found only in certain districts; by others to certain springs of water, or to the exhalations of certain marshes. The mystery attending its operations and its terrible fatality at one period created a perfect panic in the settlers; nor was this at all wonderful. The disease appears now to be vanishing."

Descriptions like this suggest inquiry into the actual prevalence of the disease in early times, and examination of the reasons for the apparent rarity of the malady at the present day.

#### PREVALENCE IN EARLY AND RECENT TIMES.

Many observations indicate that milksickness was of much more frequent occurrence in the first half of the nineteenth century than it has been during the last thirty or forty years. Thus Beach (1883),

referring to the early period of the disease in Madison County, O., declares that he "presumes nearly one-fourth of the pioneers and early settlers died of this disease." Coleman (1822) speaks of the disease being "common and unusually fatal" in a certain district in western Ohio in the winter of 1821. Compton (1881) says: "From Mr. Thomas M. Hutchins I learn that his father settled at the old fort in Dubois County, Ind., in 1815 and that more than half the deaths that occurred in that section were from milksickness. It was also very fatal among stock." Winans (1840) contrasts the status of the disease in 1840 with his early experience: "I think it probable that I had from three to five hundred patients during the whole time the disease prevailed. It has now nearly or quite vanished away." McAnelly (1836) gloomily predicts that ". . . some of the fairest portions of the West in consequence of the prevalence of this loathsome disease must ever remain an uninhabitable waste unless the cause and remedy can be discovered." Patton (1875) states: "When I began the practice of medicine in the year 1868 in Carter Township, Spencer County, Ind., I found in this disease the most formidable difficulty with which I had to contend." In the *Maryland Med. and Surg. Jour.*, 1839-40, 1, p. 133, is printed an "Extract of a letter from a physician [unnamed] of Danville, Ind., dated July 4, 1839" in which the statement is made that "Danville has now become a perfect charnel house; no less than fifty died of 'milk sick' during the past year, and that too out of a population of 500 inhabitants." Making all allowance for possible errors in diagnosis such assertions may well be considered ground for inferring a remarkable extension and frequency of the disease in parts of the central United States during the years of early settlement.

On the other hand, there is some evidence that milksickness was never so common or at all events so widespread as some of the above statements alone seem to indicate. Lea (1821) expresses himself regarding its prevalence in much the same terms that writers have used sixty to eighty years later: "In a particular part of the state of Tennessee, a febrile affection of a most curious nature is occasionally met with, which is of very rare occurrence." A good many of the earlier articles on milksickness were called forth by an outbreak of the disease in one family or even a single case; that such instances were

deemed worth reporting is perhaps significant (see, for example, Hogg, 1842; Troy, 1861). Drake (1841) who wrote extensively on milksickness devotes a lengthy memoir to the description of a trip taken by him for the purpose of studying the disease in a certain district in Ohio in which the malady was known to prevail. Many conversations with "eye-witnesses" are related in Drake's memoir, but "No case of the disease . . . either in man or brute was met with."<sup>1</sup> Sale (1871) describes the occurrence of the disease in a circumscribed location not far from Cincinnati. In this region it is said to have made its first appearance in the fall of 1818. Sale gives the following record of cases in man: 1825, 4; 1830, 6; 1834, 5; 1836, 3; 1838, 6; 1845, 2; 1856, 3; 1863, 3; 1870, 17. There are other observations that seem to support the view that the recedence of milksickness is partly apparent and is due to certain causes which will be presently specified, and that localities in which the disease may today be contracted have by no means vanished from the map. Smith (1874) of Kenton, O., in a letter to a medical journal as recently as 1874 wrote: "We are having this fall a regular epidemic of what is generally called milksickness or trembles. I have treated upwards of thirty cases." Shapard (1892) of Winchester, Tenn., expressed his belief in 1892 that the disease was then as extensive as at any time within the last fifty years. Gray (1881) in an article on milksickness notes that twelve cases of so-called gastritis, two of which were fatal, had occurred among the consumers of butter from a certain farm. Three of these had come under his own observation and were apparently genuine cases of milksickness. Gray's experience raises the question whether cases of this disease do not more or less frequently pass unrecognized and come to be reported under names such as gastritis or ptomain poisoning, thus obscuring the relative prevalence of the disease at the present time. As recently as 1907, we (Jordan and Harris, 1908) have found milksickness masquerading under the name of "alkali poisoning" in the southwestern United States where it was causing great losses among the stock and was responsible for some 38 human cases and 8 deaths in the space of ten years. According to local observers the disease has been apparently extending its bound-

<sup>1</sup> It is worthy of note that after this expedition Drake does not mention milksickness in his extensive work on the *Diseases of the Interior Valley of North America*.

aries in this region (Pecos Valley), a phenomenon perhaps connected with the introduction of irrigation facilities. We have also learned of the occurrence since 1900 of human cases of milksickness in Illinois in eight different localities in widely separated parts of the state, while prior to 1900 there are on record cases occurring in 17 localities.

Several causes have contributed to drop milksickness out of notice and even into the limbo of things forgotten. Altho the disease is by no means extinct there are now undoubtedly fewer cases in proportion to the whole population than was the case for example in the decade 1840-50. This is partly because the disease is now and apparently always has been particularly liable to appear in thinly settled regions and in marshy or timbered grazing country never brought under the plow. It is the unanimous testimony of all observers that thorough clearing and cultivation of the land will transform a dangerous area into a safe one (Drake, 1841; Crook, 1857; Pickard, 1857; Way, 1893). Drainage and cultivation have without question reduced the total acreage over which animals are liable to contract the malady. At the same time the recognition that certain tracts of land harbor the virus of milksickness has led to the adoption of precautionary measures. In many of the old milksickness districts of the Middle West pieces of land are still to be found in which cattle are not allowed to graze, and which are often very small plots fenced off from directly adjoining fields in which animals are pastured with impunity. These tracts are usually well known locally, and all the older residents have a lively sense of their dangerous character. We have ourselves inspected a number of these areas in Illinois and have been informed of many others.

Another factor that has perhaps tended to throw milksickness into the background has been the unwillingness on the part of many writers to acknowledge the existence of a specific affection bearing this title. Milksickness is not recognized in the "International Classification of Diseases and Causes of Death," and health authorities have sometimes refused to record deaths reported under the name of milksickness while accepting the term "gastritis" as satisfactory. It is our opinion that the convenient and at present fashionable refuge from etiological uncertainty which consists in referring cases of acute gastroenteritis to "ptomain poisoning" has also been made to cover



some, perhaps a goodly number, of cases of genuine milksickness. It is at least true that the symptoms that have been reported in many cases of intestinal derangement attributed to the use of milk, butter, or cheese, and that have been accredited to the ingestion of ptomaines or similar poisons are very similar to the symptoms of the disease we are now considering.<sup>1</sup> In those regions where there is any record that milksickness ever existed, cases of so-called "ptomain poisoning" or "gastritis" should be especially scrutinized (cf. Schmidt, 1877).

All these causes, the natural inability of the practitioner who has received his medical training in the centers of population to recognize at once the identity of a somewhat rare disease found as a rule only in thinly settled communities, the definite demarcation and avoidance of the infected tracts of land in all milksickness regions, and the actual reduction in the total amount of infected territory through clearing, drainage, and cultivation, have conspired to bring about the practical disappearance of milksickness from the medical literature of the last forty years. At the same time our experience indicates that in the majority, perhaps in all, of those localities where the disease has ever been endemic larger or smaller tracts of land may be found today where there is a strong likelihood that the disease will be occasionally contracted by grazing animals dependent on the pasturage for their food supply. In these localities, under conditions to be discussed later, cases of milksickness in man also occur more or less frequently. All things considered the disease has certainly not disappeared from the once-infected localities, altho it is apparently not increasing in frequency and is probably waning.

#### SYMPTOMS.

a) **Trembles in Cattle.**—Cattle are the only domestic animals in which the symptoms have been described fully and by a number of persons. There is substantial unanimity throughout the literature. The first open manifestation of the malady consists in a listlessness and disinclination to exertion. As Drake (1841) well expresses it: "The animal begins to mope and droop, and to walk slower than its fellows, to falter in its gait." Many observers have noted also the occurrence

<sup>1</sup> See for example an outbreak of food poisoning described in Michigan by Vaughan and Novy, *Ptomaines, Leucomaines, Toxins, and Antitoxins*, 1896, pp. 104-16. Symptoms of vomiting, obstinate constipation, and subnormal temperature are reported. The farm on which the cases occurred was known as "unhealthy" and previous attacks of the same kind were said to have occurred on it.



of constipation at this stage. One of the most frequently remarked symptoms is muscular weakness and trembling which makes its appearance when the animal is driven. It was the custom in many "milksick" localities for prospective purchasers of cattle to exercise the animals vigorously before consummating the purchase in order to bring out any latent symptoms. The existence of the disease in a herd was sometimes not recognized until the animals were on their way to market. Woodfin (1878) states that "Beeves that have fattened in these infected localities, started to be driven to market, take the trembles and fail the first or second day, while other cattle feeding on the same grounds, left at home, remain healthy." Tuller (1889) has described the initial stage in the following words: "The bullock when severely affected is restless and trembles as though he was receiving a shock from a galvanic battery." Johnson (1874) remarks that cattle in this stage are often greatly excited. After describing an instance in which his own son was butted down by a steer which immediately after the attack fell paralyzed to the ground, Johnson continues: "This excited condition and disposition in cattle to fight when overheated and the consequent paralysis I have often observed." Excitability, however, does not appear to be an invariable accompaniment of the disease in cattle and is not noted by the great majority of observers.

The characteristic trembling stage passes in the most severe cases into a second stage of great weakness and exhaustion. The stiffness of the joints increases and the animal after sinking to the ground is unable to rise immediately, and may remain lying for hours or days on the spot where it has fallen. Recovery sometimes occurs in animals that have reached this stage, but death ensues in many cases.

Many observers have noted the occurrence of a foul odor in animals suffering from trembles. Thus Elder (1874) refers to the "Singular fetid odor . . . pungent and corrosive . . . fetor invariably attendant upon the malady, both in man and the brute creation." J. N. Smith (1837) asserts that " . . . some of the tanners in the neighborhood can at once say, with certainty, when cattle have died of the disease, by the smell arising from the raw hides."

We had the opportunity in the Pecos Valley region in New Mexico

of studying an outbreak of the disease in a herd of about 80 cattle. Approximately 25 of this number manifested definite symptoms; 2 died, 4 were killed while moribund, and 1 was killed in an early stage of the affection. The symptoms observed were very similar to those above described. The sick animals were first differentiated from the others by their lying on the ground while the other animals of the herd were standing. On being forced to rise and walk they moved slowly and with great reluctance. If driven to trot they soon slowed down. Their gait was stiff and the fore legs were sometimes carried as if the feet were sore. When an animal was con-



FIG. 1. — Young Hereford bull in Pecos Valley, N. M., affected by milksickness.

tinuously forced it sometimes came to a stop, began to tremble violently and sank to its knees, then awkwardly came to rest with the whole body. The behavior of the young bull shown in Figs. 1 and 2 was said by ranchmen who saw it to be highly characteristic. In another instance that we observed, an eight-month calf showed when driven the typical, enfeebled, stiff gait and muscular tremors. While shambling along awkwardly it stumbled on a prairie dog-hole and lay panting, unable to rise. When we attempted to pass a thermometer per rectum the animal struggled but was held down. The thermometer had just been removed at the end of  $1\frac{1}{4}$  minutes when the animal trembled violently, took a clonic spasm in the left great muscle

of the neck and breathed in great gasps; the eyes became fixt, the mucous membranes of the mouth and nose suddenly paled, and the animal died with a few slight convulsive twitchings of the legs.

Sometimes after being disturbed the animal may have a passage of the bowels, usually of a rather thin consistency, in which may be noted much mucus mixt perhaps with blood; at other times this dropping of dung is not observed and the animal may be badly constipated. From this on, if the symptoms do not improve, the animal becomes steadily weaker, rising less often, eating and drinking less and less, until it becomes so weak that it cannot rise, but lies on its



FIG. 2.—Same as Fig. 1.

side, moaning more or less frequently, its neck often becoming twisted to one side and the neck muscles standing out on the other side, hard and stiff (Fig. 3). The sclerotics are often deeply injected and accompanied by a yellowish-red discharge which becomes gummed on the canthi. McCall (1822) and some others mention that the eyes of sick cattle are "red and suffused." The breath often acquires a more or less pronounced odor of acetone, but this odor may be absent. In the later stages of exhaustion there may be twitchings of the limbs, more particularly the fore legs, less often the hind legs, so that the ground becomes grooved by the movements. For some hours or even days before death the animal may lie on its

side with its neck outstretched and head bent back (Fig. 4). During the midstages of the disease the temperature is irregular, often ranging a degree or two below normal. We saw no cases of paroxysmal rage manifested by any of the animals we had opportunity to study. The animal dies quietly, its breathing becoming less pronounced and fitful, developing a Cheyne-Stokes rhythm; apparently all consciousness is lost shortly before death. Feces may be occasionally passed up to within a day before death, altho as a rule the animal is more or less constipated throughout the course of the disease.



FIG. 3. — Later stages of milksickness.

**b) Milksickness in Man.**—The disease has been graphically described by a number of observers, notably Coleman (1822), Graff (1841), Byford (1855), and Way (1893). The popular name of “sick stomach” draws attention to what is one of the most typical and constant symptoms of the malady, namely, prolonged and violent vomiting. Obstinate constipation is also a practically invariable accompaniment of the disorder, and is given a prominent place in all the early descriptions. Thompson (1853) records the interesting observation made upon several patients whom he treated that “as soon as even moderately free action of the bowels was obtained, a dysenteric character was manifested in the stools.” Associated with



this derangement of the alimentary tract is great thirst, but the water taken is usually vomited again immediately. Prior to the appearance of these more active symptoms there is usually a period of weakness and increasing debility. Cosby (1866) states that the first stage in some instances lasts for eight or ten days before the second stage sets in. According to Logan (1849) the disease sometimes never gets beyond the first stage: "There are two forms in which we meet with the disease; the acute and subacute or chronic. To the first the name of milksickness is usually given and to the latter the appropriate



FIG. 4.—Later stages of milksickness.

title of *slows*. They are, however, the same disease produced by the same cause and each liable to be transformed into the other and differing only in degree. In the subacute form the individual is languid, unable to make any exertion of body or mind, appetite variable, bowels rather torpid, palpitation of the heart, some degree of stiffness of the limbs, trembling and sickness of stomach if any considerable exertion is made or if taking food is deferred beyond the usual time. This stage of things may exist for months after the cause is once introduced unless removed by the sanative efforts of nature or by a proper course of remedial treatment, or it may be transformed into the acute form by long fasting, fatigue, or overexertion, and in some instances

by neglecting the bowels or suffering them to remain constipated for some days.

"When the disease assumes the acute form, the individual is seized suddenly with nausea, faintness, and prostration; surface and extremities below the natural temperature—sometimes cold and clammy; great distress and anxiety depicted in the countenance and the bowels almost universally constipated."

Headache is sometimes noted, but this does not seem to be a usual symptom. Coleman (1822) mentions the occurrence of "pains or soreness in the calves of the legs," and Graff (1841) and others also state that patients complain of pain in the limbs. Abdominal pain is generally entirely absent and the abdomen is flat and flaccid. Johnson (1866) thus describes the condition seen by him: "In a few hours from a healthy condition of the functions of the bowels the most obstinate constipation sets in, and so completely are they paralyzed that not the least movement of them can be observed. I have seen this condition last for a week or even two weeks without a solitary observable peristaltic movement, without pain or swelling or tenderness to pressure."

McCall (1822) and a few others state that fever is one of the accompaniments of milksickness, but the great majority of observers declare that the temperature is below normal during most of the course of the disease. Simon (1875) states that fever exists, but contradicts himself in a later paper (Simon, 1888). Way's statement may be said to represent the general consensus: "The temperature is never elevated; usually it is subnormal, ranging during the course of the disease from 97° to 98° F. At times it may be even lower than 97° F., and a return to normal or, as not infrequently happens, a slight elevation of one or two degrees above normal occurs coincident with improvement in cases that recover" (Way, 1893).<sup>1</sup>

Nearly all writers mention a peculiar odor emanating from the breath of the patient or observable in his neighborhood. The odor has been variously described as "garlicky," "like chloroform liniment," "sweetish," etc. Tuller (1889) in discussing this odor says: "An undertaker who has had a great deal of experience in burying those that had died of the disease, in speaking to me of this peculiar odor,

<sup>1</sup> See also Pusey (1880).



said it reminded him of the smell of rising bread in making milk or salt-rising bread." Byford (1855) compares it to a mixt smell of chloroform and mercurial salivation. Many writers regard the peculiar odor as a highly characteristic feature of the disease and one on which they would be willing to base a diagnosis. It seems to be essentially similar to the odor noticed in cattle (Elder, 1874; and others).

The pulse is slightly increased or may be slightly below normal; respiration is normal but becomes rapid on exertion. The urine is scanty with a trace of albumin, hiccough is sometimes noted as a distressing accompaniment of the latter stages. Eye and knee reflexes are normal.

Some writers express skepticism as to the existence of a definite affection which should be known as milksickness. Yandell (1852) who wrote rather voluminously about the matter concludes one long article with the opinion that "the various accounts of this disorder nullify each other; and the mind is left in extreme doubt whether there is anything specific in milksickness." Anderson (1867) goes so far as to assert: "I consider 'milsick,' as the vulgar call it, a mere matter of credulous fancy." Other writers such as Chapman (McCall, 1823), Thompson (1854), and Bowen (Bailey, 1868) simply declare their disbelief in the existence of milksickness. Against these *obiter dicta* are opposed the opinions of the great majority of observers who hold with Waggoner (1858) that "no one that has ever once seen a case of the disease can fail in making a correct diagnosis."

Six cases of milksickness in man have come under observation by one or both of us, two near Carlsbad, New Mexico, and four in Altamonte, Ill. None of the five resulted fatally. Through the kindness of Dr. W. E. Walsh of Morris, Ill., we have also seen several cases occurring in November, 1908, in his practice and described by him in a separate publication (Walsh, 1909). All presented some of the typical features of milksickness as recorded in the early literature, namely: (1) slow onset with rather long prodromal period of weakness and debility; (2) excessive vomiting; (3) obstinate constipation; (4) no fever, but normal or subnormal temperature; (5) slow recovery with prolonged muscular weakness and disinclination to exertion. Two of the five cases first examined, one in each group, had the

characteristic odor of breath so frequently referred to in the descriptions of the disease. The odor seemed to us to be like that of acetone. The urine of one of these patients (A. McC.) was examined and gave a positive reaction for acetone (Legal's test). In the Morris outbreak samples of urine were obtained through the courtesy of Dr. Walsh and were kindly analyzed for us by Dr. R. T. Woodyatt of the Department of Chemistry in this University.

Specimens I and II were respectively from Mrs. J. (Walsh case, No. 3) who was quite ill at the time of voiding the sample, and from Mr. J. (Walsh case, No. 4) who had recovered some few days previously from a mild attack of the disease.

Specimen I: In two containers; both samples showed the same characteristics and after preliminary tests were mixt: what follows applies to the mixt specimens.

Clear, yellow, alkaline, odor of acetone, albumin absent, reducing substances absent, acetone present as shown by the following tests:

a) Urine has the characteristic odor.

b) Urine + solution of iodine in a KI solution when treated with a strong KOH gives a strong odor of iodoform with a precipitate of characteristic pale color, which when viewed under the microscope is seen to be composed of hexagonal crystals.

c) 50 c.c. of urine diluted to 250 c.c. and treated with 3 c.c. of a 10 per cent solution of  $\text{H}_2\text{SO}_4$ , distilled until 25 c.c. only remained. The distillate smells of acetone. A small portion gives the iodoform test. The entire distillate remaining treated with a solution containing

Paranitrophenylhydrazine.....	3	gm.
Hydrochloric acid.....	0.8	"
Water.....	10.0	"

gives partly in the cold, completely on warming, a mass of long, fine, silky, golden, thread-like crystals which after washing in cold water and drying over  $\text{H}_2\text{SO}_4$  show a melting-point of  $148^\circ\text{C}$ . accurately.

Another portion of this urine was similarly treated with  $\text{H}_2\text{SO}_4$  and distilled. The distillate was then made alkaline and redistilled gave the same results as in the foregoing. (This proved the neutral character of the substances isolated in both instances as opposed to, say, formic acid which is also present in the urine of this case.) The crystals correspond to the paranitrophenylhydrazone of acetone (see Bamberger and Sternitzki, *Ber. d. deutsch. Chem. Gesellschaft.*, 1893, 26, p. 1306).

From 50 c.c. of the urine was recovered 0.25 gm. of the hydrozone; this corresponds to 0.083 gm. of acetone, equivalent to 1.6 gm. per liter of the urine, i. e., a total daily output of 2.4 gm. supposing that the patient had passed 1,500 c.c. of urine of the same kind as the specimen examined.

d) The urine also shows with  $\text{Fe}_2\text{Cl}_6$  a marked "bordeaux red" coloring which disappears with heating and with mineral acids (e. g.,  $\text{HCl}$ ). Heating the urine for five minutes at the boiling-point before applying the test practically destroys the test, especially after making the urine acid. The color fades spontaneously when the test has stood for a few hours: result, *aceto-acetic acid*.

e)  *$\beta$ -oxybutyric acid*.—A sample of the urine saturated with ammonium sulfate and made acid with  $\text{H}_2\text{SO}_4$  (10 per cent solution), then shaken with ether several

times; the ether evaporated and the residue taken up in water and polarized shows marked levo-rotation of 0.7 degrees. The unconcentrated urine also shows a levo-rotation of 0.22 degrees.

f) Indican, phenol, etc., not found in increased amount above that found in ordinary clinical tests.

Specimen II: This sample presented the following characteristics: alkaline, heavy deposit of phosphates, no albumin, no reducing substances, no surely demonstrable acetone, no aceto-acetic acid or other abnormal constituents.

An incomplete examination of urine of fatal case H. J. (Walsh case, No. 2) showed:  
Albumin—none.

Reducing substances—none.

Acetone—odor, and iodoform test positive.

In six of the seven cases reported by Dr. Walsh a sweetish odor was noted in the breath. Several of the patients complained of pains in the calves of the legs and in the joints, and also of burning epigastric pain. One case showed a brief period of active delirium. In these cases the liver areas were percussed out, and in two the liver was found projecting below the margin of the ribs (2.5 cm. and 4.5 cm. respectively). In the third case (convalescent) the liver could not be felt below the margin of the ribs, but the attendant physician informed us that enlargement of the liver had existed prior to our examination. Dr. F. F. Doepp of Carlsbad, N. M., who has attended a good many cases of the so-called alkali poisoning has stated to us that the liver is enlarged in all cases seen by him. One of the striking features in all the cases we have observed was the very great exhaustion and muscular weakness which persisted far on into the convalescent stage. Great stress is laid on this feature in the descriptions of early writers.

c) **Other Animals.**—Besides cattle, many other domestic animals are said to be affected with a disease resembling trembles. The symptoms, for the most part, are not given with any degree of fulness, but are described as “similar to those of the disease in cattle.” Horses, sheep, goats, and dogs are all said to suffer from the disease under natural conditions. Hogs are declared by some writers to be susceptible to the disease (McCall, 1822; Drake, 1840; Davis, 1881; Coleman, 1822; Winans, 1840) while by others they are asserted to be rarely affected or entirely immune (Woodfin, 1878; Johnson, 1866). The available evidence seems to be in favor of the view that hogs are not as liable to contract milksickness as other domestic animals, but that nevertheless they are not under all circumstances

exempt. Chickens, buzzards, vultures, and crows are also said to be affected by feeding on the carcasses of animals dead from trembles.

Statements are made that various wild animals are sometimes affected. Among those mentioned by several different writers are deer, wolves, and foxes.

McCall (1822) describes with some detail the disease in a dog: "I saw a dog pass through every stage of the disorder. He had fed on the flesh of a calf which died by sucking its mother's milk. The dog coming home with some putrid flesh upon him, I suspected what he had eaten and watched him. He appeared to be in a state of great anxiety—went to a spring for water and then returned to the poisoned carcase. Calling him he came to me manifestly very sick. On the way he ate some grass and vomited. After getting home he concealed himself under a floor where he lay all the ensuing day. His eyes became fiery red, and a peculiar hoarseness marked his barkings. He remained in the same condition next day, refusing every kind of sustenance except water. His whinings were almost continued, and he seemed intent on biting whatever came within his reach. The next morning I found him dead."

A good many other writers mention the susceptibility of the dog, but give no description of symptoms (Sykes, 1891; Woodfin, 1878; Davis, 1881; Buck, 1840; Johnson, 1886). A suggestive note is made by Travis (1840) to the effect that his "own dogs have had the disease in such a manner as to render them unable to walk, by feeding on a dead cow." Drake (1841) says that affected dogs drop dead when fighting or running.

We have ourselves seen no dogs naturally affected with the disease, but we have been informed by reliable witnesses in and about Carlsbad, N. M., that dogs have been sometimes observed to sicken and die after feeding on the carcasses of "alkalied" animals. It is, however, certain that the flesh of such animals will not invariably communicate the disease. In New Mexico we several times made the experiment of feeding dogs with considerable quantities of meat (thigh muscle) from animals dead of typical trembles, but in no case did we succeed in producing any symptoms of illness.

We have not ourselves seen cases in sheep, but the following notes were made for us by Mr. D. D. Todd, Fellow in Bacteriology, who

under our instruction investigated an outbreak of the disease in sheep in Tiro, O., in June, 1908.

**Lamb 1.**—Male, age 10 weeks. The animal when first seen was lying on its side, made no attempt to get up, and did not resist being handled. It showed no sign of pain, but occasionally ground its teeth. The eyes were clear and there was no injection of the sclerotics. Axillary and inguinal glands were not palpable. Breathing was regular, 28 per minute; pulse, 118. Some mucus in nose.

**Lamb 2.**—Female, age 10 weeks. This animal, unlike the first, often struggled to get on its feet, but was unable to rise. It would after struggling keep its legs and feet going in a walking motion for some seconds. Breathing was forced and jerky, but regular, rate 33; pulse, 111. Autopsies were made on both these animals (see p. 449).

We have seen one horse (in New Mexico) which may or may not have been a representative case. We were informed by ranchmen, however, that "alkalied" horses usually showed the symptoms observed in this animal. This animal had been out on pasture land on which two other horses had contracted the disease and had died a day or two before this animal was seen. The horse stood in its stall, legs somewhat wide apart, and motionless except for its somewhat rapid breathing, sweating profusely about the head, neck, and shoulders, back, and flanks. Its head hung low and the animal appeared to be fully conscious. Its breath possessed a faint odor of acetone. Its rate of breathing was 28 a minute and its pulse 72. On percussion, the lung note was fairly clear and no sign of any pneumonia or bronchopneumonia could be discerned. Auscultation was rendered difficult and untrustworthy by the squeaking of the hair on the hide during the breathing, but no blowing note or coarse rales could be made out. No muscular tremors were noticed while the animal stood in the stall. The feces which had been passed were well formed and did not look abnormal. The animal died at midnight the next day after having been ill about 72 hours. It is to be regretted that no autopsy was obtained on this animal but circumstances rendered this unavoidable.

#### INCUBATION.

By far the majority both of isolated cases and family outbreaks of milksickness are described as ushered in by a period of indefinite length during which the patients "did not feel well." Williams (1863) expresses the general belief when he states that "for several days previous to the attack the patient has a general feeling of indis-



position." According to Way (1893) the onset of milksickness is never sudden or violent. In a few instances a definite period of incubation has been supposed to precede the appearance of violent symptoms. Beach (1883) records what he considers a case of probable incubation of two days and Cosby (1866-67) one of less than 36 hours. Buck (1840) reported a unique family outbreak, Case I developing "in September," Case II on October 17, Case III on October 19, Case IV on October 21, and Case V (Buck himself, the attending physician) on November 10. The second, third, and fourth cases were in persons associated with or attending upon the first. If it be granted that these cases were really cases of milksickness, which seems somewhat doubtful, the instance stands practically alone, since other recorded family epidemics indicate simultaneous or nearly simultaneous infection of the different members and there is no evidence pointing toward contact infection. Spalding (1881) notes one instance of an interval of three days and another of six days between the discontinuing of suspected milk and the onset of symptoms. McCoy (1907), who investigated a recent epidemic in Tennessee, altho able to establish the date of infection with a high degree of probability, could not learn the exact dates when symptoms appeared except in the case of one guest in whom they appeared "about ten days after he had taken breakfast at the house."

A number of writers have put on record cases where intoxication rather than infection seems to afford the most plausible explanation for the sudden onset of the symptoms. Most of these are attacks of disease attributed to the use of meat, altho McCall (1822) states that symptoms appear "in a short time" after swallowing milk. Coleman (1822) personally observed "an instance of a whole family becoming sick with this disease, some of them in a few hours after dining upon a loin of veal, in which it was afterwards satisfactorily ascertained that the calf labored under the disease at the time it was butchered." Lewis (1829) mentions one family outbreak in which 17 persons were "all attacked with the same disease in a few hours after using the milk of cows which had fed in a milk-sick district of country, some more and others less violently." The same writer records another case where immediate effects followed the eating of steak; "Mr. T. expired at the table while eating (!), his wife soon



after." Drake (1841) advances the following somewhat doubtful evidence: "In the month of July last, about twenty of the boarders, in the hotel of Mr. Madeira, Chillicothe, O., were attacked, in one, two, or three hours after breakfast, with nausea and vomiting. . . . Of course, this affection was ascribed to something eaten at the table, but the only article taken by the whole was butter; and that butter, it was ascertained had been brought from an adjoining county in which the milksickness prevails." Still more dubious, perhaps, is the outbreak of food poisoning reported by Barbee (1840): "In March, 1838, a family of six persons, traveling westward, put up at a house a few miles east of Terre Haute, Ind. At breakfast they all partook freely of *butter* and *milk* and departed immediately on their journey. By the time they reached Illinois, in five or six hours, they were all taken with nausea, vomiting, etc., and died, every one of them, in from two to six days. Upon inquiry it was ascertained that the place where they had taken their breakfast was in a 'milk-sick' region." There is no convincing evidence that either of these outbreaks had anything to do with milksickness. Sale (1871) enters into greater detail: "A young calf took the trembles and died; the cow was milked and the milk fed to two pigs that were in a pen; these pigs thrived and fattened, and in due time were slaughtered. The first meal that the man and his wife (owners of the pigs) took made them sick and they both died in four days after eating the pork, with all the symptoms of milk sickness. *Neither cow nor pigs showed any signs of the disease.*" Altho recorded more circumstantially it should be noted that the instance quoted was not put on record until twenty-five years after the event (1845).

Some of the most definite symptoms of milksickness are lacking in these instances of sudden onset, and while it is possible that the ingestion of poisonous compounds formed by *B. lactimorbi* may account for some of these outbreaks, it may be questioned whether such cases do not in part belong to other varieties of food poisoning. Our own experience has been entirely with cases showing a longer or shorter period of *malaise* prior to the appearance of severe symptoms. In one of the New Mexico family epidemics we have obtained a full and clear account of the circumstances surrounding the outbreak from an observer of good judgment who was himself one of those who

suffered from the disease (A. M. H.). There seems in this case reason to suspect the flesh of a calf slaughtered about December 1, 1906. So far as can be gathered active symptoms appeared in four different individuals within 7 to 10 days after beginning to partake of the meat of this animal, and a fifth case had "a touch of the sickness" about four weeks later. In each instance a period of indisposition preceded the attack. In the Altamonte, Ill., outbreak studied by us, we were informed that the four youths who were affected had been feeling "out of sorts" for about a month prior to the onset of violent symptoms. In this outbreak, as in many others, infection was probably more or less continuous over a considerable period. Similar conditions prevailed in the Morris, Ill., outbreak (see p. 416, also Walsh, 1909).

#### RELAPSES. IMMUNITY.

One of the most characteristic features of milksickness is a tendency or at least a liability to relapse or exacerbation. Logan (1849) states that the subacute forms of the disease "may exist for months" or "may be transformed into the acute form by long fatigue, fasting, or overexertion and in some instances by neglecting the bowels or suffering them to remain constipated for some days. Especially if the patient takes vigorous exercise during convalescence the symptoms are apt to recur. Reagan (1884) expresses the general consensus when he says, "It (the poison) lies quiet in the system for a long time without exhibiting any of its toxical powers until the person or beast is forced to take exercise." Genuine second attacks are, however, reported. McAnelly (1836) records an instance where a farmer and his wife were attacked by the disease "every summer," and adds, "I was called to see them in August, 1833, in their fourth attack and I do not recollect ever having seen a more violent one." Spalding (1881) mentions a case of man and wife who had a first attack in April, 1876, and a second in July, 1878, and refers to another instance of a second attack 10 years after the first. Gray (1881) also reports an instance of relapse. Jones (1852) cites the following remarkable case as occurring under his own observation: "A Mr. Taylor and his family, who had contracted the disease in a 'milk-sick' district in the mountains of North Carolina, and who had been relieved afterwards, settled in the neighborhood where I was born (southwestern Virginia)

where 'milk sickness' was never known to prevail for many miles around. They remained in the neighborhood and appeared to enjoy excellent health for about two years. In the autumn of the second year, the father, mother, and two brothers were again seized with symptoms of 'milk sickness;' the disease assumed a violent character . . . [all died]. Not another case occurred in the whole county either of man or beast."

We have had no personal experience of successive attacks in human patients, but all observers with whom we have talked are agreed that the liability to relapses is one of the marked features of milk sickness. Dr. F. F. Doepp of Carlsbad, N. M., who has seen a number of cases of the disease<sup>1</sup> during the last 10 years, informs us that recovery is usually slow and that if great care is not used the patient is likely to suffer a relapse, especially after overfeeding or overexertion.

It is the unanimous testimony of stockmen that cattle do not acquire immunity from an attack of this infection. We ourselves saw one fatal case in a heifer which was said by the owner to have shown symptoms of the disease the preceding year.

#### MORTALITY.

Statements in regard to the mortality differ greatly. Coleman (1822) asserts that "about one case out of twenty or thirty is lost of those who experience the disease in its active form." Allen (1878) places the mortality about twice as high as Coleman. Hibberd (1844) thinks the mortality about 2 per cent of the whole number attacked and about 5 per cent of those claiming medical assistance. Tuller (1889) estimates a mortality of 10 per cent, while Collins (1902) says, "I find that about 40 per cent of my cases in which I am reasonably certain of the diagnosis have died."

The list on p. 425 gives those instances we have been able to collect in which the number of cases and deaths is specifically stated.

Through the kindness of Drs. Friedman and Doepp of Carlsbad, N. M., we have obtained a record of 38 cases and 8 deaths occurring in and about Carlsbad in the period 1898-1908. We have also been informed in a personal letter by Dr. R. J. Boatman of Carlsbad of 7 cases and 2 deaths that occurred in his knowledge in Nashville, Ill., in 1864. Of 4 cases investigated by us in Altamonte, Ill., in 1908 none

<sup>1</sup> Locally known as "alkali poisoning."

resulted fatally. There was 1 fatal case and 6 recoveries in the Morris, Ill., outbreak in 1908. Adding the cases obtained by us to the numbers given below, we have a total of 318 cases and 75 deaths or a mortality of about 20-25 per cent.

Cases	Death	Authority			
4	2	Lewis (1829)	6	5	Graff (1841)
10	5	Carney (1847)	9	5	Tuller (1889)
1	0	McNutt (1847)	2	0	Scott (1889)
49	26	Sale (1871)	6	1	Hurd (1875)
50	7	Smith (1867)	13	2	Sykes (1891)
30	0	" (1874)	6	1	Lyday (1896)
3	2	Houser (1880)	9	1	Michigan S. B. H. (1897)
22	4	Spalding (1881)	5	2	Collins (1902)
3	1	Gray (1881)	13	6	Palmer (1904)
4	2	Simon (1888)	6	6	McCoy (1907)
11	6	Pusey (1880)			
			262	64	

There is no doubt that the real mortality from the disease is much lower than this. Many of the cases reported are in family outbreaks which attracted notice from their severity, and it is undoubtedly true in this as in many other diseases that the milder or "abortive" cases are regarded as less worthy of report and often do not come to the notice of physicians at all. We believe it safe to assume that the mortality from milksickness does not exceed 10 per cent and may be considerably lower.

#### SOURCES OF THE DISEASE.

a) **In Cattle and Other Grazing Animals.**—All observers are practically agreed that cattle pastured on particular tracts of land acquire the disease, while at the same time others on adjoining pastures never show any signs of illness. A few illustrations may be cited from the voluminous literature.

"A farmer on the high and dry land near Annapolis, Ind., was in the habit of keeping a portion of his cattle overnight in a small lot, in which was no water and but little vegetation. Those cattle were attacked with sloes or milksickness, while the cattle on other portions of the farm were exempt. He excluded his cattle from the lot, and the disease never occurred on the farm afterward" (Wilkinson, 1857). Shelton (1836) relates an instance where by fencing out live stock from a suspected tract of ground the disease was suppressed for eight or nine years. Then, "the enclosure having decayed and fallen down,"

the disease again manifested itself "and rarely fails to occur in animals that feed long on the spot." The same writer states that "in Blount County, Tenn., there is a locality embracing not more than ten or fifteen acres, on which the disease has been known to originate for nearly, perhaps quite, forty years." Drake (1841), as the result of an investigation of the conditions under which the trembles occurred in southwestern Ohio, drew the following conclusions: "We feel warranted, then, in deducing and resting upon the following conclusions: (1) That in *this district*, the trembles in cattle, horses, sheep, and hogs, are produced by their frequenting the densely timbered table-land, which from its flatness abounds in wet places and ponds, indicated by the presence of lofty white elms, black walnuts, maples, burr oaks, and other trees, which delight in a rich and moist soil; (2) that when the same animals frequent prairies, barrens, and the hill-lands, near the larger streams, although they may be heavily timbered, they do not contract it; and consequently, its cause does not exist there, or at least is not efficient."

Crook (1857) asserts that, "In no instance has it ever been known in this county [Spencer County, Ind.] during any other than the grazing season, and then *only* upon lands that had never been in cultivation." Many other writers claim that stock kept on tame or cultivated pastures never contract the disease, and that only wild lands can give rise to the affection (see Bennett, 1822; Pickard, 1857; Simon, 1888; *et al.*). Walker (1886), however, relates the following case: "Dr. W. S. Sims of this place tells of a farmer in Hamburg Township, Jackson County, N. C., who has a half-acre lot enclosed with his dwelling. In this enclosure are fruit-trees and some of the native grasses, and the place has been under cultivation for twenty years or more, and yet whenever cattle are turned upon that lot during grazing season they are sure to die with the disease in a few days." The explanation of this apparent exception or anomaly is perhaps given by Shapard (1892) who declares: "I am not alone in the opinion that there are fields in this county [Franklin County, Tenn.] that have been in cultivation a long while, perhaps 50 years, in which the farmer does not feel entirely safe in allowing his cattle to roam or graze at certain seasons of the year." In such cases Shapard believes that the persistence of the disease on cultivated lands is due to the



existence of some strip of woodland, rocky knoll, or fence corner, which has not been reached by the plow.

There is little or no doubt that clearing, drainage, and cultivation of land greatly diminish if they do not altogether do away with the liability of grazing animals to contract trembles. The connection between a special locality and the occurrence of the disease is in any case well established. Sawyer (1867) expresses the general opinion when he asserts, "It does not appear to extend its limits, but where it now is it *always existed*." And there is practically no observer of this disease who would not agree with Nichols (1876) when he says, "I can point to a certainty to every district or farm where this disease does or does not prevail."

The complete exemption of certain pasture lands whose topography and vegetation are apparently identical with those of the "milsick" areas has led many to believe that the disease is closely associated with the soil itself. This opinion is strengthened by such observations as those of Allen (1878), who writes: "There are certain localities in that county [Effingham County, Ill.] where cattle resort for the purpose of licking. They lick large holes in the earth and when that is the case many of them die of the disease."

Our own observations completely confirm the existence of a connection between this disease and particular soil areas. We have seen in New Mexico and in Illinois adjoining tracts of land apparently identical in topography and vegetation, the sole discoverable difference being in their ability to cause trembles in grazing cattle. Many small pieces of pasture land in the Middle West remain to this day fenced off from the rest of the farm on account of their dangerous character, demonstrated or suspected. Some of the cases of milksickness in man reported in recent years have followed the change of ownership of "milsick" farms and the disregard by the new owner of the warnings given him by better-informed neighbors. In all instances the localization of the disease has been one of its most striking features.

Not only soil in particular localities but water has been suspected of harboring the exciting agent. Beach (1883) mentions an instance where cattle contracted the disease after eating hay from a meadow that had been overflowed with water draining from a tract of land notorious for producing trembles in grazing cattle. Lyday (1896)



states as an uncontroverted fact that (in North Carolina) "the disease is sometimes contracted by drinking the water of the streams which run out of the 'milk-sick' coves." White (1836) gives several specific examples, not, however, any too well authenticated, of outbreaks among sheep and cattle attributed to drinking-water. Fulton (1884) records the following cases in which water seemed the probable or only possible source of the disease. After quoting some hearsay evidence, he says: "Many similar instances could be cited, some two or three of which have fallen under my own observation. One in Richland Township, Logan County, O., where the cattle had been taking the disease from the first settlement of the country, and several persons on that and adjoining farms had taken it, and most of them had died, until a spring was finally suspected and inclosed—since which time to the present, some fifteen years, there has been no more of it.

"Another, that of a low, marshy piece of ground in Brunswick Township, of the same county, the country around which was notorious for the number of cattle that died of the disease, and of persons that took it, until, with the improved agriculture of the country, that place, with other low lands, was ditched and drained; since which time there has been no more of the disease in that neighborhood in either man or beast.

"Another instance, that of a stock-well on a farm in Clark County, as I am informed by several credible witnesses, from which the cattle were accustomed to be watered, and had been taking the disease for a number of years, when it was suspected and closed, since which time there has been no more of it.

"Again, there is a farm in Hardin County, on which members of every family that has ever lived there have had the disease. On that farm I have attended patients who said that they had not used meat, milk, butter, or cheese for weeks, guarding against the disease. The presumption therefore was that they, and also those of the previous families that had lived there, had obtained it directly from the water." No attempt to discredit the evidence for occasional water transmission appears to have been made, altho Tuller (1889) states that he has never known the disease in cattle or man to be caused by drinking-water.

b) **In Man and Other Animals.**—The circumstance that has given this disease its name of *milksickness* is well authenticated. There are numberless instances on record where the use of milk from particular cattle has been followed more or less promptly by the characteristic symptoms of the malady. Some of these have been already cited (p. 421). Townshend (1883) describes the following family outbreak which came under his own observation.

“Of a family of six persons, attended by the writer, five, who had used the milk of the same cow, had Milksickness; the sixth person used neither milk nor butter and escaped entirely. At the time of the illness of this family a yoke of oxen belonging to them were sick of trembles and both died. The cow which furnished the milk used by the family and which had pastured with the oxen was at the time severely sick, but finally recovered.”

McCoy (1907) has recently reported a somewhat similar case in Marion County, Tenn., “So far as could be learned the cow responsible for the outbreak had obtained access to what is locally known as ‘poison ground’ about the 10th of April.

“The milk from this cow was used regularly, and apparently without bad results, until April 25.

“In addition to the five members of the family, there were two guests at breakfast on that day. Of the seven persons who ate breakfast on that date (April 25) all used milk and butter, with the exception of the mother of the family, and all who used the milk and butter became sick and died.”

Other writers presenting more or less cogent evidence of the causation of the disease from drinking raw milk are McCall (1822), Lewis (1829), Barbee (1840), Logan (1849), Philips (1857), Cosby (1866), Nichols (1876), Woodfin (1878), Sykes (1891), and Beck (1905). In fact there seems to have been no doubt in the minds of all the early settlers that milk was the article of food clearly incriminated. Instances of this belief are found in the works of early travelers through the West as well as in medical journals. M’Kenney (1846) relates the following experience: “A settler came to my encampment (1827) [about 18 miles above St. Louis, on Mississippi River]. I asked if he could supply me with some milk. He answered, ‘We don’t use it.’ I asked why. ‘The people,’ he replied, ‘about these parts, were

afraid of the *milk-sick*; and never used milk after early spring. They do not even permit the calves to suck it; if they do, the calves die, as well as the people.' ”

Long (1823) makes a somewhat similar statement. It has already been pointed out that in nearly all the districts in which milksickness has been observed the cattle suffer from the malady known as trembles or slows. An anonymous writer in the *Western Medical Gazette* (1832) expresses the prevailing opinion when he writes: “I have never known nor indeed ever heard of the milksickness prevailing where the cattle were not subject to the trembles.” While this seems to be generally true there are some alleged exceptions which will be considered presently. Our own observations upon the connection of milk with the disease in man are limited to an outbreak in Altamonte, Effingham County, Ill., in the summer of 1908.<sup>1</sup> Four cases of the disease appeared in a family of ten living on a farm known from the days of earliest settlement to give rise to milksickness. A particular spring and the creek fed from it had long been suspected as the source of infection in cattle. The disease, however, had not been known to occur in early summer, and cattle were accordingly usually pastured on that portion of the farm where the spring was located. They were kept there in 1908 in the early season until about June 22. About 10 days later acute symptoms of milksickness made their appearance. The whole family partook regularly of the milk, cream, and butter derived from these cows that had been pastured in the neighborhood of the suspected spring, but those individuals that manifested symptoms<sup>2</sup> were said to have taken particularly large quantities. Both parents escaped altogether; the elder daughter who had remained well had been away from home prior to the outbreak; the younger daughter was said to have taken little milk or butter; a boy of nine remained entirely well and a child of two and one-half years showed no characteristic symptoms, altho vomiting had occurred while the others were ill. The cows were to all appearance entirely healthy, and showed no signs of disease before or after the outbreak; a six to eight-weeks-old calf whose mother was one of the animals providing the milk used in the family also seemed in the best of condition. A

<sup>1</sup> We are under obligation to Dr. F. Buckmaster of Altamonte for notification of this outbreak and for other kind and effective assistance.

<sup>2</sup> Four young men, aged, respectively, 22, 20, 16, and 14.

cat that was being fed on the milk from these cows was itself well, but lost all its eight-weeks-old kittens, five in number, during the outbreak in the family. A dog receiving milk from the same cattle showed signs of illness and was noticed to vomit repeatedly. The vomit was a clear watery fluid; no acetone odor to the breath of the animal could be detected.

In the Altamonte outbreak it was not possible to differentiate between butter and milk as possible factors in causing the disease. Butter, however, has been regarded as in itself capable of causing milksickness. Johnson (1866), Scott (1889), Beck (1905), and others speak of the agency of butter in the production of milksickness as if it were well demonstrated. Gray (1881) records 12 cases of "gastritis," believed by him to be milksickness, which occurred during the space of six years in families using butter made on a particular farm. Beach (1883) records a specific case in which the patient "ate some butter on his bread, but no cheese, milk, or meats of any kind." Nichols (1876) makes the statement that "man receives this poison through milk, cream, or animal food and *never* by butter," but later in the same paper adds the interesting qualification that "butter may produce trembles if churned from sweet cream, otherwise it never does."

Any doubts that we may have had about the relation of butter to this disease were set at rest by an outbreak coming under our observation at Morris, Ill., in November, 1908.<sup>1</sup> This, like most of the recorded cases, was a family outbreak, a fact that in itself goes far to fasten suspicion upon some particular article of food or drink. At the time the cases appeared (five in a family of six), there were no other cases of the disease in the immediate neighborhood. The eldest son of the family (J., 17 years) was employed on a farm (P.) about six miles from Morris; this young man brought butter from the P. farm to the J. family, but no milk. The butter was churned from the milk of cows that had been allowed to forage during the fall of 1908 in some woods long known as a locality in which grazing cattle were likely to acquire trembles and in which eight cattle had contracted the disease and died in 1906. At the time of

<sup>1</sup> We are greatly indebted to Dr. W. E. Walsh, of Morris, who kindly notified us of this outbreak and has assisted us in its study in a variety of ways.

the present outbreak the cows on this farm showed no signs of illness. About three weeks before the cases developed in the J. family, two young men on the P. farm had complained of feeling "out of sorts" and very weak; one of them "could not climb into a wagon;" this lad about a month later, and after the J. cases in Morris had recovered, came down with a typical case of milksickness apparently as the immediate consequence of a hard day's labor husking corn. In this patient there was present the typical acetone odor to the breath and urine as well as other characteristic symptoms of milksickness. It may be added that the youth J. who worked on the P. farm and, like the P. sons, drank the milk of the P. cows, remained free of any symptom of illness. Two or three hogs, however, fed with milk from the suspected cows, are said to have died with symptoms resembling those of trembles.

There seems little doubt, therefore, in view of all the facts, that butter brought from a "milksick" farm was responsible for the cases in the J. family in Morris. It should be remarked that in this outbreak as in many recorded in the early medical journals the milk and milk products of animals themselves apparently healthy proved capable of giving rise to fatal illness in man.

It has been supposed that, in addition to butter and milk, cheese is sometimes the source of milksickness. Some writers (e. g., Scott, 1889) refer to cheese as if it were a generally recognized carrier of the specific poison, but we have yet to find any particular instance in which the epidemiological relation of cheese to milksickness is clearly made out.

On the other hand the flesh of animals is definitely implicated as the bearer of the disease-producing agent. Carnivorous animals are alleged by many writers to acquire the disease from feeding upon the carcasses of animals dying from trembles. Many of these statements seem to be based on direct, first-hand observation (see, for example, Coleman, 1822; De Bruler, 1858). Dogs, pigs, and wolves and also fowls, buzzards, and crows are asserted by numerous observers to contract the disease in this manner. Specific instances of the causation of the disease in man from meat-eating are given by Coleman (1822), Lewis (1829), Sale (1891), and others. Conner (1904) records the case of his father who died of milksickness when he himself was



eight years old. "My father and two companions took dinner in a restaurant in Springfield, eating fresh beef, and in a few days two of them were dead and the other almost dead, recovering after a lingering illness." McCall (1830) cites an outbreak where some sheep died from trembles and were eaten by hogs which in time perished from the same malady. The carcasses of the hogs were burned except one which was accidentally overlooked and was picked by chickens. The chickens were used for food and were supposed to have caused milksickness in five persons, two adults and three children; one son "who ate none of the chicken or soup was not sick." Johnson (1866) also declares that "persons who neither eat milk, butter, or beef have taken the disease and died from eating chickens that had it." Various other writers refer to the causation of the disease in man from eating the flesh of beef cattle, calves, hogs, or chickens as if the circumstance were well authenticated.

We have ourselves seen two cases of the disease in New Mexico in which inquiry did not bring to light any indication of milk or butter transmissions, while a possible origin was found in meat eaten at a Chinese restaurant. In a severe family outbreak occurring about a year before our visit, the trouble was attributed to meat. We are fortunately able to give a full description of the circumstances surrounding this outbreak in the words of the head of the family (A. M. H.), a man of unusual judgment and insight.

About December 1, we butchered a calf that had had the run of the yard around the place. The weather turned warm and the meat began to spoil. My wife did not tell me about this, but tried to save as much of the meat as possible by trimming out carefully the tainted parts. We hardly ever use the liver and kidneys, but the girls had found some new recipes that they wanted to try and used both the liver and kidneys.

Our boy, the youngest child, was on a milk diet exclusively. He showed no signs of illness. One of my nieces ate very little meat at any time and had hardly touched the meat in question. She worked night and day for three weeks in caring for the rest of us without getting sick. The other niece had eaten some of this meat, and had a touch of the sickness. Our two little girls that died were always helped at table by me and no doubt got the same poison as I did. My wife also ate meat liberally.

Towards the last of November cattle came to my place for water. Many of them died afterwards from "alkali." A Mexican had his team around the place more or less; shortly afterwards one horse died from the same disease as the cattle. During this time it rained more or less and pools of water stood in many places in the yard, and I saw this calf drink from these pools several times, though it had access to good water. The calf had all the skim milk it wanted and was in fine condition.



Attention has already been drawn to the possible rôle of water in causing the disease in cattle. In man also there is some evidence of water transmission. McAnelly (1836) records several cases where the occurrence of the disease on a particular farm seemed to be connected with the use of a well water. The same writer adds, "The disease sometimes prevails in the human species in districts where the inferior animals are comparatively exempt and frequently where none of them are known to be affected at the time, as in the neighborhood of Johnstown, O., where the inhabitants use water from the wells while the stock get theirs from the brook before spoken of." Taylor (1842) also attributes a particular human case to drinking mineral water "at the very place most of our cattle and horses have died." Brewington (1876) makes the following interesting statements: "I do not believe that in this vicinity there is one in fifty persons that take the disease who gets it by using milk or butter from diseased animals. In places where the disease used to be most prevalent, the people used water from wells for cooking and drinking, but since they built cisterns and use water from them exclusively, they are entirely exempt from the disease."

Sager (1879) relates a specific case considered by him to be certainly caused by drinking-water: "April, 1878, was called to see McK.'s wife. He was a poor man, lived in a rented house, and no well water to use, consequently got their water to drink and for culinary purposes out of a ditch which contained clear water, but this ditch drained timbered land also. I found Mrs. McK. very sick with all the symptoms present peculiar to milksickness. Upon inquiry they informed me that they drank no milk, neither ate butter, as they had no cow and were too poor to buy the milk and butter. Neither did they eat any fresh meat. In both cases my conclusion was drawn in favor of the third proposition (water), being satisfied that heavy dashing rains will remove the germs from their fixed places and carry them into pools and ditches."

Pusey (1880) describes a series of cases very striking from the point of view of possible water infection: "It is claimed that all the patients got the disease on the Tate farm, which is situated half a mile north-west of the farm on which the Clarke family died. In July and August Tate's wife and two children died and three others recovered, all

patients of Dr. Pennington of Benleyville. After the death of Tate's family the Clarkes bought his growing crop and the place was vacated till October; then the Clarkes went on the premises to dig the potatoes and to make sorghum. While engaged at this work four of them got sick so nearly together and so nearly alike and immediately after drinking of water that had stood overnight in a bucket near the cane pile, as to impress them with the idea that they had been poisoned by the water. Three of the Clarkes died and two recovered.

"About sixty years ago, as I learn by tradition, and within the memory of one person living in the vicinity, a family of the name of Hull, living on what is now the Tate farm, all died from milksickness. The water of two springs was believed to have contained the poison."

Conner (1904) mentions a case of supposed water transmission which, however, did not fall under his own personal observation.

In the limited number of cases of this disease that have come directly to our own knowledge we have not met with any that it seemed reasonable to ascribe to drinking water.

A few instances of supposed cutaneous infection are recorded. An anonymous physician writes from Indiana to the *Maryland Medical and Surgical Journal* (1839) as follows: "The late Professor John Eberle told me, when I visited him a year or two since, that he attended with Dr. Drake on two cases in Cincinnati, laboring under 'milk sick.' It appears that the men had driven a cow from Lafayette, in Indiana, to Cincinnati, where it died, and they determined to take off the animal's skin, being the only part of the animal which was available. One of them had cut his hand a day or two previous, and the other, in skinning, cut his finger with the knife, and four days after they were both seized with 'milk sick' and one of them died."

Sale (1871), apparently giving the results of his own experience, states that "in 1836 a cow died of the disease; two men skinned her and a woman rendered the tallow. They all died of the disease two days after." Other writers (see Elder, 1874) express the belief that one way in which the disease may be produced is "by skinning animals that have died of the malady." Hibbard (1844) on the other hand, is incredulous as to the disease having been contracted by skinning carcasses. In those localities where we have had the opportunity of studying this affection, no evidence of cutaneous infection has

presented itself. Ranchmen and others have testified that they frequently opened the bodies of animals dying from trembles without experiencing any ill effects, nor have they known of any case of the disease in others contracted in this manner.

#### THE NATURE OF THE VIRUS.

a) **Behavior towards Heat.**—According to one observer (J. N. Smith, 1837) the active agent in or upon the soil is destroyed by heat: "About fifteen or twenty years ago, we are told that the disease was particularly severe in the edge of an adjoining county, and from some accident during a dry time in autumn the woods became ignited and burned over a circumference of several miles; and in the following autumn, either by accident or design the burning was repeated. Since which time there has never been a case of the disease in that neighborhood. And the inhabitants do not even think it necessary to keep up their cattle."

Yandell (1841) and Chesney (1880) mention that it was the custom to burn the carcasses of animals dead from trembles. C. H. Smith (1867) gives an interesting account of an outbreak observed by him: "All cases that occurred here were in American families, there not being one in a German family. Now the cows of the two classes run in the same ground and the cattle of both die with the disease; yet I have never known a case to occur among the Germans. The reason is, I think, plain. The Americans use the milk just as it comes from the cow, and the Germans boil what they drink."

Johnson (1874) is very emphatic concerning the action of heat in destroying the virus: "Heat also destroys the poison, whether in the milk, butter, or flesh, the opinion of Professor S. H. Dickson to the contrary notwithstanding. I have enquired of a great number of people living in various milk-sick regions as to whether cooked beef was ever known to impart the disease, and the invariable reply was, no."

Sager (1879) thus expresses his conviction: "What is the poison? . . . . We answer, fixed low organisms, and that fire destroys them. . . . The above has been thoroughly tested by the farmers in the vicinity where I live. Mr. John Smith, Sr., and others have cleared pieces of ground with the timber standing of all the old logs and

rubbish lying on the ground and consumed the same with fire. Since that, the same ground has been pastured with horses, cattle, and sheep with impunity."

One small family outbreak in Carlsbad, concerning which we have obtained first-hand information, affected two members of the family who drank raw milk; a third member who drank the same milk boiled escaped. Graff (1841) states that butter after being heated retains all its virulent properties, altho he also affirms that the extract obtained by boiling meat for several hours has no perceptible effect even when given in large quantities. Crawford (1908) has called attention to the discrepancy of Graff's statements.

b) **Persistence in Soil, Hay, etc.**—Under certain conditions the virus of milksickness may persist in the soil in a given locality for at least fifty or sixty years (Pusey, 1880) and probably for even longer periods. It is true, however, that as a rule such milksick tracts of land remain able to communicate the disease only if they are left in a wild or uncultivated condition. As long ago as 1822 Bennett wrote: "Those farmers who keep their cattle and horses in inclosed pastures which have been cultivated, do not lose them by this disease." The same opinion is expressed by Crook (1857), Simon (1888), and many others. Clearing, drainage, and cultivation of a milksick area destroys its dangerous quality. Pickard (1857) reports the following case: "Another family having suffered from its ravages, plowed up a pasture field, digged around the stumps, thoroughly turning all the soil, and sowed the field in grass, upon which they have kept their stock for 20 years, and at no time has milksickness made its appearance; while on other portions of the farm uncultivated, it is as fatal as ever." Drake (1841) in his elaborate memoir on Milksickness concludes that "clearing and cultivation, even girdling the trees, harrowing the ground, and sowing it with grass seed, destroys or renders inactive the cause, whatever it may be." In a letter received by us from Dr. Morrow B. Wilson of London, O., we are informed that farmers in that locality still find that drainage and cultivation of milksick areas destroy the virus in the soil; and cutting out the underbrush and letting in the sunlight is equally efficacious.

According to Gray (1877), Beach (1883), and others, the virus may be gathered and stored away in hay. Beach says: "That cattle

*may* have trembles in the winter season, is a matter of occasional observation; and the old citizens generally attribute it to the feeding of cattle upon wild or swamp hay." The same author gives a specific case of trembles apparently due to hay cut from a meadow overflowed by drainage from a milksick locality. One writer (White, 1836) makes the following statement on the persistence of the virus in meat " . . . one case occurred within our knowledge in the course of the last summer which was brought on by eating dried beef that had been slaughtered the preceding fall."

c) **Effect of Season.**—A difference in the seasonal incidence of the disease has been noted by many observers. Drake (1841) states that "it occasionally occurs in May and June, but its usual time of prevalence is August, September, October, and November." Dawson (1842), Shapard (1892), and many others refer to the more extensive prevalence of the disease in late summer and autumn than at other times of the year. Rawlings (1874) maintains that the "cause . . . is affected by the change of season in the same way that malaria is." While in most localities an autumnal incidence is noticed, this is not invariable. Beck (1857) refers to its having prevailed in Kentucky exclusively in winter while in the locality from which he wrote (Cadiz, Ind.) it prevailed "in all seasons except winter." Beck adds, "If we have abundance of rain in the spring, with hot sunshine alternating with thunderstorms and growing showers, as we sometimes have the last of April and during May and June, then we confidently expect milk sickness in May and June. A dry spring and summer will postpone it till September or October." Dewey (1854) speaks of the disease as probably due to "a succulent plant" bearing a white blossom. According to this writer the plant in question is destroyed by the first hard frost "when it and the disease contemporaneously disappear." There is, however, abundant testimony that the disease occasionally appears in winter (Yandell, 1852; Beck, 1857; Waggoner, 1859; Beach, 1884; Shapard, 1892). In the Pecos Valley region the disease generally occurs in November and December, but we were told on reliable authority of some cases that occurred in March. We have seen human cases in Illinois in early July and in late November.

Altho there does not seem to be an exact and invariable correspond-



ence between the season of the year and the disease, an autumnal incidence exists in most localities. This is one of the chief reasons for the frequent comparison of milksickness to malaria made by authors, and for the ascription of a "miasmatic" origin to the former malady. On the assumption that the virus of milksickness has its habitat in the soil, cattle might be supposed to contract the disease more readily at the season of the year when the pasturage is scanty and the herbage is consequently cropped closely. Many statements confirming this explanation might be cited in addition to those already quoted. One writer (Sager, 1879) makes the statement that sheep are more liable than other animals to contract trembles because of their habit of close cropping.

It seems to have been a common belief among farmers in certain milksick regions that cattle kept "up" till the dew had evaporated were less likely to develop the disease than if allowed to go out to pasturage in the morning early (Evans, 1860; Walker, 1886, *a*). An oft-quoted statement from Reagan (1884) also bears on this question: "It is known by all who live in the vicinity of the poison that the danger is when the dew is on vegetable matter, if it were vegetable in its origin it would poison at any time of the day, which is not the case. In order to test this matter they took hay from a settlement remote from this knob and let it lay out on the knob during the night and fed it to a healthy cow while the dew was on it. Again they tried the same and waited until the sun had thoroughly dried the hay, then fed it to a cow and it did her no harm." It seems possible to explain such observations as Reagan's by assuming that microorganisms present on the hay were killed by drying or sunlight. As regards the alleged influence of dew in favoring the production of disease it is evident that particles of soil (containing microorganisms?) cling more readily to moist blades of grass than to dry herbage. Woodfin (1878) suggests that the influence of dew is only apparent and that the reason why cattle in some regions seem to contract the disease more readily if turned out to pasture early or brought home late is that they are able to range farther and so to reach the affected, and usually remote, tracts of land. Davis (1881) would explain the influence of dew by the fact that: "Animals eat more greedily when the vegetation is saturated with water than they do when the vegetation is dry."



One of the most remarkable features of this disease is its power of self-propagation. As already shown, the flesh of cattle dying from trembles will sometimes if eaten communicate the malady to other animals. If the accounts of numerous eyewitnesses are to be believed, the flesh of these animals in turn will give rise to fresh cases and so on with seemingly no limitation. Sale (1871), for example, records an outbreak in which the virus was passed from a cow to pigs and from the latter animals through the pork to man, while neither cow nor pigs showed any signs of illness. DeBruler (1858) relates an occurrence which fell under his own observation: A cow gave the disease to her calf which died and was eaten by two dogs; both dogs died with the usual symptoms of "tires;" a pet crow fed on the dogs' flesh also died. McCall (1830) cites one instance of the passage of the virus from sheep to hogs, and hogs to chickens. The chickens were used as food and caused milksickness in a man and wife and their children; one son "who ate none of the chicken or soup was not sick." Philips (1877), whose observations seem to have imprest him strongly with the infectious character of milksickness, declares with emphasis that the disease is "transmissible and reproduces its kind." Perhaps the most definite statement on this point is made by Graff (1841) who says: "This subtle poisonous principle, of whatever it may prove to consist, seems to possess the power of infinite reproduction, by some vital or chemico-vital action of the system of those animals poisoned by its influence. Thus, suppose one pound of flesh to prove sufficient to produce the death of another animal, it will be found that each pound of flesh of that animal so destroyed, will possess as active powers of destruction, and will, in its turn, serve to contaminate the whole body of another animal in the same degree. I had wished to repeat this so often as to demonstrate the possibility of its reproduction *ad infinitum*. In this I failed, from the difficulty experienced in compelling 'dog to eat dog.' From what I have observed, I would say that there exists a power of generation of the poisonous principle in the animal economy. This primary impulse being received from the specific action of some substance, vegetable or mineral, by some means obtained from the soil."

Our own observations and experiments upon the transmissibility

of this disease will be considered more fully in another connection. It is sufficient to mention here that we have at various times fed several dogs (four) with the flesh of animals dead from trembles and have observed no ill effects. This corresponds with the irregularity in feeding experiments noticed by some other observers. Whatever be the cause, feeding does not act uniformly and constantly.

**d) Contributing Factors.**—One circumstance often remarked is the exemption of certain cattle in a stricken herd. On this point Coleman (1822) says: "Cattle and horses do not always become afflicted with the disease if allowed to range in the woods where the disease is common;" and similarly Woodfin (1878): ". . . many animals may feed on the same lands and evidently partake about equally of the poison and some of them suffer violently and die; others suffer lightly and recover, and others still, perhaps the larger number, remain quite healthy; and that the same is true of families containing several persons." Gray (1879) mentions a family outbreak where the parents had the disease and a child subsisting on milk from the same cows showed "not the least symptom of the poison." Perhaps some of the differences observed are due to differences in the quantities of virus taken into the system, others doubtless depend upon variations in individual susceptibility.

There is no doubt that violent exercise is a predisposing factor and has much to do with the development of acute symptoms. "The people in the infected districts very often chase their beef cattle around with dogs until they are heated by the exercise; then if the cattle do not show any signs of the disease they are killed and eaten as beef without fear" (Davis, 1881). Dixon (1833) says: "It is one of the peculiarities of the disease that the poison occasionally lies long dormant in the system, and only becomes active when excited by some indiscretion or violent bodily exertion." Woodfin (1878) states that "beeves that have fattened in these infected localities, started to be driven to market, take the trembles and fail the first or second day, while other cattle feeding on the same grounds, left at home, remain healthy; and dogs eating the flesh of milk-sick cattle, by lying quietly during digestion, remain healthy, while others put on the chase, or otherwise overheated, soon sicken and die." Reagan (1884) asserts that, "It (the poison) lies quietly in the system for a long time without

exhibiting any of its toxical powers until the person or beast is *forced to take exercise*." Another writer (Simpson, 1839) expresses his opinion that the causal agent may "exist and lie dormant" in the system for a day and finally pass off without harm "unless it is developed by some exciting agent." He also adds that the prominent exciting causes of the disease in man are "over-exercise and excess in drinking spirituous liquors." According to Logan (1849) a subacute form of the disease "may exist for months after the cause is once introduced unless removed by the sanative efforts of nature or by a proper course of remedial treatment, or it may be transformed into the acute form by long fatigue, fasting, or over-exertion and in some instances by neglecting the bowels or suffering them to remain constipated for some days." The practice of bleeding patients with this disease is said by Simpson to be highly injurious. Sale (1871) has reported the interesting observation that "when phlegmonous erysipelas attacked the face and scalp in the cases reported, all the grave symptoms, such as distress at the pit of the stomach, vomiting, constipation, etc., subsided."

**e) Elimination of Virus in Milk.**—The opinion has been expressed by several writers that when the virus of this disease is eliminated in the milk, the affected animal is in some measure favorably influenced by such elimination. It is even asserted that "cows giving milk never suffer from the disease, the poison being eliminated by the milk" (Johnson, 1874). Other writers express themselves in less sweeping terms: "Cows giving milk do not manifest strong and decided symptoms" (Jackson, 1881). On the other hand there are numerous instances reported where milch cows have died from the disease after their milk had been observed to give rise to the malady. Some of them have been cited elsewhere in this paper.

#### POSTMORTEM EXAMINATIONS.

There are few autopsy records giving any but the most meager details or bearing the stamp of any wide experience. The brief descriptions are for the most part couched in general terms. The stomach contents of cattle are said by one observer to be "dry" and he adds that "the liver has a morbid appearance" (Bennett, 1822). According to another, "In every case where animals who have died

of this disease have been dissected, the heart and aorta have been found greatly enlarged and their inner coats sloughy" (Beck, 1822). Another says vaguely that the disease "is attended with gastric and hepatic lesions" (Miller, 1867). Rawlings (1874) states that the "liver has usually been found congested with blood of a dark color." Hibberd (1844) mentioned one condition which "obtained, I believe, in all the subjects that were examined, whether of the human or brute family, viz.: *a diminution of the caliber of the stomach and intestine.*" Davis (1881) mentions a case in which "the opposing internal surfaces of the small intestine at one place were united by adhesive inflammation for three or four inches of its length." Mendenhall (1861) also notes the evidence of change in the alimentary tract: "The stomach and intestine are inflamed; the mucous coat is measurably destroyed; the entire coats are in some cases gangrenous." Home (1843), who made several autopsies, states that the alimentary tract is always inflamed either as a whole or in part and that the mesentery likewise is inflamed, and the mesenteric glands enlarged. In one case "the liver was of a natural size, but looked very pale; its peritoneal lining presented in two or three places an inflammatory blush." Newman (1867) records the following unique observation: "In examining the animal (a milch cow) after death, the skin was found very much thickened and covered with small pustules." No other observer seems to have noted such a condition.

A few more detailed descriptions are scattered through the literature. Dickey (1852) describes a postmortem made 10 hours after death in which the peritoneum showed marked signs of inflammation. The small intestine also showed evidences of severe inflammatory action, particularly in the jejunum and ileum where "the mucous coat was easily separated by the finger nail." Peyer's patches were ulcerated, the spleen was enlarged, and "there was ramollissement of the substance of the liver." The kidneys were healthy. McClelland (1854) describes in somewhat similar terms an autopsy upon the body of a robust laborer made eight hours after death. This writer says that the liver was enlarged and softened, and that "evidence of inflammation existed through the entire course of the small intestine." Wayman (1841) says of a case examined 15 hours after death that there was not much emaciation, that the colon was dark

brown with rose-colored patches, and that the liver was of a dark color and seemed unusually friable under pressure by the finger. He also states that the gall-bladder was much distended with a black pitchy bile, and that the spleen was much enlarged, of a deep brown color, and very soft. The peritoneum had reddish spots and there was some increased effusion into its cavity.

Preston (1843) describes an autopsy made 13 hours after death. The skin was brownish-yellow. The omentum major was in the first stage of inflammation; the liver was enlarged and the gall-bladder adherent to the ascending colon and distended with dark bile. The stomach and duodenum were inflamed, the jejunum and ileum "inflamed in various patches." Preston further describes the pancreas as being "twice as large as natural," the mesentery highly congested, the mesenteric glands enlarged, and the kidneys enlarged and softened. The lungs were said to be apparently pneumonic.

Carney (1847) reports a postmortem examination in the following words: ". . . internal coat of the stomach, particularly the lower portion, gangrenous; whole intestinal canal exhibiting a highly inflammatory state; liver healthy except the small portion embracing the bilious, tar-black and hardened gall-bladder enlarged and filled with black unhealthy matter; stomach and bowels about empty; heart and spleen healthy."

Graff (1841) describes two autopsies made on dogs and one on a human subject. These are often quoted and have been made the basis of most of the statements upon the pathology of this disease that have found their way into textbooks and general treatises. Graff states that in one dog the intestines were much diminished in caliber and the stomach greatly contracted. The arachnoid was minutely injected and there were other evidences of cerebral inflammation. In another a similar condition of the stomach and small intestine was observed, the spleen was enlarged and the liver softened. "The lining membrane of the large intestine was nearly natural in appearance." Examination of the brain and cord showed marked evidences of meningeal inflammation. In an autopsy made upon a woman "by stealth at night, in the open air, and by the light of a single candle" Graff found likewise diminution in the caliber of the small intestine and stomach and profound inflammatory disturbances of the central nervous system.



The liver was said to have a peculiar appearance. These descriptions by Graff differ from those hitherto cited in their insistence upon severe inflammation of the brain and cord. Whether this is because Graff's cases were peculiar in this respect or simply because no examination was usually made of the central nervous system is uncertain.

Way (1893) in a brief note on morbid anatomy mentions having observed congestion of the abdominal viscera in "the lower animals" and in one case of a six-weeks-old calf intense congestion of the cerebral meninges.

Our own observations comprise examinations of several cattle, two lambs, one horse, and one human case.

a) **Cattle.** MACROSCOPIC.—All our observations on bodies of cattle dying from this disease were made in the vicinity of Carlsbad, N. M., in the autumn of 1907.

Externally the body presents nothing on inspection that is to be considered worthy of note. On reflecting the skin of the animal one can often detect an odor of acetone, particularly if the animal has just died. There are no subcutaneous lesions of any sort to be found.

On opening the cavities of the thorax or abdomen the odor of acetone is usually very marked.

*The thoracic cavity.*—Occasionally there may be a small quantity of clear yellow fluid present in the pleural cavities, which looks like serum and clots quickly on exposure to the air. There is no pleuritis. The *lungs* do not collapse greatly on opening the thorax. Several small areas of atelectasis may be found scattered over the dorsal aspect of the organs or at the apices of the upper lobes; at times one notices an occasional small area beneath the surface of the pleura which gives the impression that the part is filled with blood. The color of these organs varies at different times from a normal salmon color to a generalized purplish red, the latter condition being always found after a relatively long period of illness. The lungs feel boggy and on section exude a great deal of frothy fluid, i. e., they are in an edematous state, and this circumstance accounts for the incomplete collapse of the organs on opening the thorax. Sections of the most edematous portions, however, float in water. We did not on any occasion find any traces of bronchopneumonia or lobar pneumonia. The mucosa of the trachea and bronchi showed no signs of inflammation. No ecchymoses were found on the pleural surfaces.

*The heart.*—As a rule the pericardial sac contained varying quantities of clear straw-yellow fluid (50 to 175 c.c.) which clotted on exposure to the air. Pericarditis was not met with, at least such as could be laid to the disease; in one instance the visceral pericardium was thicker than normal, giving a uniform grayish tone of color to the whole of the organ—this was evidently due to some old attack of pericarditis. The right heart was generally found distended with blood, whereas the left side of the organ was usually found empty. Among the most noticeable things seen on opening up the pericardial sac were numerous areas of ecchymoses beneath the visceral pericardium, distributed chiefly along the course of the cardiac veins from base to apex and at times



numerous at the base of the heart around the roots of the great vessels. These areas varied in size from that of a mere pin-point to irregular areas measuring approximately 2 by 2.5 mm. and the accumulation of blood sometimes bulged the serous covering up above the surface about 1 mm. in places. In some of the animals ecchymoses were of slight extent and in one case absent. No ecchymoses were ever seen on the parietal surface of the pericardium. As a rule the heart muscle was always definitely paler than normal, sometimes giving the impression of the existence of cloudy swelling, and at other times showing small areas of fatty metamorphosis. On opening the heart there were never present any evidences of the existence of endocarditis. In two cases ecchymoses were found beneath the endocardium over the tricuspid valves close to their bases, the accumulation of blood bulging up the endocardium about a millimeter high. On section the musculature showed the same appearances as seen on the outside, namely, either fatty areas or streaks, or more or less generally cloudy swelling; this was brought out particularly on making sections of the muscle in the left ventricle, the apparent fatty areas being well marked.

*The abdominal cavity.*—On opening the peritoneal cavity there was in general little or no fluid present above what might be considered normal. No evidences of peritonitis were ever seen. In some cases small scattered areas of ecchymoses were found beneath the parietal peritoneum; these never measured over three square millimeters; more rarely were such found beneath the visceral peritoneal coat. The stomachs were usually well filled with food material in contradistinction to the intestines, both small and large, which were usually empty. The appearance of the small intestines, particularly in the duodenal and jejunal portions and less so as the large gut was approached, was that of a general and deep injection of the vessels beneath the peritoneal wall and within the walls of the gut itself. The large gut, as a rule, was in an apparently normal state and occasionally contained some fecal material.

*Liver.*—The most conspicuous condition pathologically among the organs was the state of the liver. Uniformly this organ was much enlarged, of a purple red color, being full of blood, with at times areas or streaks showing yellowish against the red color of the rest of the viscus. The gall-bladder was most often full of bile of a dark green color. On removing the liver from the body and applying pressure to it, the dark red color disappeared and was replaced by that of a reddish yellow. The consistency of the liver substance was much diminished and it required little pressure of the fingers to produce rupture. At times an occasional ecchymotic spot could be seen under the capsule. On section the organ dript considerable blood and exposed a reddish yellow surface, the presence of the blood vessels everywhere being plainly marked out, giving the typical appearance of the "nutmeg" liver. The parenchyma was markedly fatty. The gall-bladder in some of the animals on being opened showed a small degree of injection of its mucous coat; at other times it looked quite normal. The bile was always clear and of a dark greenish color.

*Spleen.*—The spleen, so far as determined, was little, if any, enlarged in any of the cases, the capsule often being wrinkled up and thick and of a pearly gray color. In two of the animals the spleen showed several small subperitoneal ecchymotic patches, but as a rule these were absent. On section the organ was of a deep maroon red color, and the Malpighian bodies were not seen at all distinctly, sometimes not at all. The fibrous tissue framework was in all cases very evident. The consistency of the spleen was thought to be normal in all cases, at least it was not particularly soft.

*Kidneys.*—The kidneys, so far as we were able to judge, were always enlarged and

engorged with blood, having in consequence a deep brown-red color. In certain of the animals the kidneys showed a definite cloudy swelling of the parenchyma, and in one instance slight fatty change. On section the organs contained a very considerable quantity of blood. The Malpighian tufts always stood out plainly on the cut surface. On the elimination of the blood from the organs in certain of the cases the appearance of cloudy swelling or of fatty metamorphosis seen in the organ before removal from the body vanished so that it could not be determined with certainty whether there existed any degenerative changes or not.

*Bladder.*—The bladder often appeared on the outside deeply injected, but this condition was not constant. On opening the viscus the mucous surface was at times found to be injected, at times not. There were no ecchymoses seen under the mucosa. In certain of the cases the urine contained within was cloudy and apparently contained traces of blood. The adrenals seemed to be normal.

*Intestines.*—On opening the small intestines they were seen to be much injected in the mucous membrane and also showed here and there in some of the animals small ecchymoses in the mucosa. As a rule the gut in the duodenal portion held a considerable quantity of greenish yellow mucus mixt in small amount with material from the stomach. Passing downward through the jejunum and upper parts of the ileum, the mucus in the gut was of a very tenacious character and of a definite yellow color, with little or no food material in it; it clung to the walls of the gut strongly and was mixt with small bubbles of gas. The large gut on being opened appeared normal in all the animals.

*Muscles.*—The general musculature of the animals was normal in appearance, being not pale or of deeper tint than usual; no ecchymotic spots were ever found beneath the muscle sheaths or in the muscle substance.

*Brain.*—In only one animal was the brain exposed, and this only with difficulty on account of lack of proper tools. In this case there was some congestion of the meningeal vessels, but no redness of the parenchyma of the brain. On section the ventricles of the brain appeared also congested and there was an excess of fluid, which was not, however, cloudy or blood-stained.

**MICROSCOPIC. P. B.**—Three-year-old Hereford bull, shot in head with rifle while in an early stage of the disease (Fig. 1). Tissues preserved in formalin; stained with hematoxylin and eosin.

*Heart.*—Left ventricle: Muscle fibers have for the most part lost their striations, are fibrillated, nuclei frequently swollen, vesicular, and do not stain well.

*Lung.*—Hyperemia; some atelectasis; some alveoli are filled with serum, others with red blood cells. No bronchitis; no bronchopneumonia.

*Liver.*—Hyperemic; cloudy swelling in moderate degree, affecting the liver cells here and there in the columns; slight amount of fatty change in some parts of the section.

*Kidney.*—Hyperemia; glomerulitis present as shown by the swollen cells of the capillary loops and the apparent increase of nuclei, and much coagulated material in Bowman's capsule. Parenchymatous degeneration of a mild degree present in the convoluted tubes more particularly; much granular material within the lumina of many of the tubules.

*Spleen.*—Hyperemic only.

*Duodenum.*—Much round-cell infiltration of the mucosa and hyperemia; epithelium everywhere intact.

**P. H. 1.**—Two-year-old heifer—pregnant; autopsy five hours after death. Formalin preservation; hematoxylin and eosin.

*Heart.*—Left ventricle: A moderate degree of parenchymatous myocarditis present; striation cannot be discerned, many nuclei pale and distorted and absent; muscular substance in places granular. On cross-section, fat droplets can be seen within the cells.

*Lung.*—Hyperemia; atelectasis in certain portions; in others edema which affects some of the smaller bronchioles as well as the alveoli, some local emphysema. No bronchitis or bronchopneumonia.

*Kidney.*—Hyperemia; a mild grade of parenchymatous degeneration of the convoluted tubules and of the glomeruli.

*Spleen.*—Hyperemia; altered blood pigment in some cells of the pulp; otherwise normal.

*Adrenal gland.*—Hyperemia.

*Pancreas.*—Hyperemia.

*Liver.*—Hyperemia. Parenchymatous cells swollen and filled with large and small droplets of fat; some few nuclei degenerating. No cholangitis; bile ducts flattened out with the pressure of the swollen liver cells.

*Stomach.*—Fourth division (three sections): All normal. Fourth division (pyloric end): With the exception of two small foci of round-cell infiltration in the deeper part of the mucosa, the specimen is normal.

*Small intestine.*—Acute enteritis: loss of columnar epithelium from the villi; invasion of the mucosa by round cells; enlargement of the solitary follicles with occasional necrotic areas within the follicles; in one of the follicles resting on the inner muscular coat of the bowel is a partial section of a possible vermicular parasite in a coiled attitude, and having an apparent chitinous covering; it lies in a smooth-walled cavity. Edema of the submucosa and general hyperemia.

**Fetus** (about four to five months old).—Formalin; hematoxylin and eosin.

*Liver.*—Cloudy swelling of the liver cells is the prominent feature of the specimen; in many cells, however, the nuclei stain pretty well altho the protoplasm barely stains at all; some of the cells contain fat droplets. The capillaries are filled with blood and show a high degree of round-cell accumulation in which the sizes of the individual cells differ considerably, and in many areas they are found massed together; in the blood also can be found a relatively large number of large, round, polynuclear cells which take a deep chromatin stain and whose protoplasm stains well with eosin (Engel's metocytes?). Bile ducts are seen in many places.

**P. H. 2.**—Young heifer, 10 hours after death. Formalin preservation, stained in hematoxylin and eosin.

*Heart.*—Left ventricle: Parenchymatous degeneration, fatty metamorphosis in certain bundles of fibers; much finely granular brownish pigment noted in the muscle cells in some places.

*Cortex Cerebri and Cerebellum.*—Appear normal.

*Lung.*—Generalized edema, with a few scattered alveoli air-containing. Occasional areas of atelectasis. Interlobular tissue edematous. Hyperemia. No bronchitis or bronchopneumonia; no pleuritis.

*Kidney.*—Acute diffuse glomerulonephritis; in certain of the collecting tubules are seen some polymorphonuclear leukocytes. In two localities of the section are areas of acute interstitial nephritis.

*Spleen*.—Hyperemic, otherwise normal.

*Liver*.—Generalized cloudy swelling accompanied by a moderately high grade of a fatty metamorphosis, the fat occurring in small and large droplets. The parenchymatous degeneration is most marked in the middle and inner zones. Pressure of the swollen cells partially obliterates the capillaries and smaller bile ducts, altho the central veins are well filled and prominent.

*Adrenal gland*.—Hyperemic, but otherwise quite normal.

**P. H. 3.**—Young heifer, moribund, killed by blow on head, autopsy at once. Formalin preservation; hematoxylin and eosin.

*Heart*.—Left ventricle: Slight evidences of cloudy swelling in some of the muscle bundles accompanied with fatty changes. Striation is indistinctly present in a few places.

*Lung*.—Hyperemia; some atelectasis; no inflammatory reaction anywhere; no edema. Another section: Hyperemic; considerable atelectasis, and in these areas there appears to be some multiplication of connective-tissue elements. The walls of the alveoli are everywhere thickened by increase of their connective-tissue cells. No inflammatory reaction of bronchi, or of neighboring tissue, or of interlobar septal tissue.

*Kidney*.—Moderate degree of diffuse glomerulonephritis; some of the tubules hold granular material and a few red blood cells.

*Adrenal gland*.—Normal.

*Pancreas*.—Normal.

*Spleen*.—Practically normal, there being some dark brownish pigment scattered about throughout the specimen.

*Liver*.—Nearly all lobules show cloudy swelling distributed in the middle and inner zones accompanied by fatty changes. Capillaries are indistinct in many places, due to the pressure of the enlarged liver cells; central veins full of blood. No cholangitis.

*Stomach*.—First division (two sections). Normal.

*Small intestine*.—Enteritis, small-cell infiltration, loss of columnar epithelium and in some places partial destruction of the Lieberkühn's follicles. Hyperemia.

**Calf 1 (Galton's).**—Fell suddenly dead while being chased. Formalin; hematoxylin and eosin.

*Liver*.—Cloudy swelling accompanied by a high grade of fatty metamorphosis; capillaries and smaller bile ducts compressed by the enlarged liver cells. Central veins widely open and filled with blood.

**b) Lambs.**—The two lambs previously mentioned were chloroformed while in a moribund condition and their bodies examined immediately. The following notes were made:

**Lamb 1.** SECTION.—On reflection of the skin there was no sweetish odor noticed. Superficial blood vessels were not injected. Inguinal and axillary glands were not found enlarged. Abdominal cavity: There was no peritonitis and no excess of fluid found. The stomach was very full; small intestine empty and of a deep red color; the large intestine contained a few fecal masses and appeared normal. The omentum was in a normal state. The mesentery was studded with small hemorrhagic areas or ecchymoses, measuring 1–3 mm. in diameter; there were a few small hard thrombi found in the mesenteric veins. The mesenteric glands were not enlarged. The liver

<sup>1</sup> The naked-eye observations upon the bodies of these animals were made by Mr. D. D. Todd, Fellow in Bacteriology.

was of a very dark red color and filled with blood; the under surface showed a normal appearance, except the quadrate lobe which showed a marginal hemorrhagic area  $2 \times 8$  mm.; the diaphragmatic surface of the organ presented on its left lobe four small whitish patches about 1 mm. in diameter not elevated above the surface, and looking somewhat like scar tissue. On pressing the liver the blood could be locally driven out and the tissue took on a decidedly yellow appearance, and was very friable, the changes could be described as fatty metamorphosis. The gall-bladder was very full but showed no inflammation. The spleen was of a bluish white color and hard to the touch; on section the Malpighian bodies could not be made out. The kidneys were both apparently normal; the adrenal glands were likewise. The retroperitoneal glands were not enlarged. The urinary bladder was full and the vessels were injected. On opening the small intestine its contents were of a frothy yellowish mucous character; no nodules or other peculiarities were noticed. The stomach was filled with grass and the mucous membrane of the four portions was seemingly in a normal condition.

*Thoracic cavity.*—The lungs were collapsed, of decidedly reddish color, and showing many hemorrhagic areas measuring from 1 mm. to 2 cm. in width; these were most commonly present at the roots of the lungs. Apart from the presence of scars on the pericardially opposed surfaces of both organs and the occurrence of the hemorrhagic areas the organs were in a relatively normal condition. The heart appeared normal. Pericardial sac and membranes in a normal state. Blood in the heart was markedly laky.

*Brain and meninges.*—Brain matter was very soft. In the posterior part of the superior longitudinal sinus was found a small thrombus. Meninges normal and there was no excess of fluid beneath them.

**Lamb 2.**—Female, age 10 weeks. This animal in contradistinction to the first often struggled to get on its feet but was unable to rise, and after such struggles it kept its feet and legs going in a walking motion for some seconds. Breathing was forced and jerky but regular at the rate of 33 per minute. Pulse 111 per minute.

**SECTION.**—No palpable glands observed beneath the skin. No odor of a sweetish nature on reflecting the skin.

*Abdominal cavity.*—Considerable peritoneal fluid present altho there was no peritonitis present. The small intestine was in greater part injected deeply; a few isolated areas 15–30 cm. appeared normal; these latter areas felt thicker to the touch than did the injected areas. No nodules were found on the external surfaces. The mesentery contained numerous hemorrhagic spots as did that of Lamb 1. The venous system of the mesentery was full. The mesenteric lymph glands were much enlarged and elongated. The large intestine was injected, contained very little fecal matter, and was filled with gas. The liver was soft, friable, filled with blood which on being pressed out in parts showed a yellowish color to the tissue; fatty changes were manifest; there were several hemorrhagic areas found on the under surface of the liver. The spleen was bluish-white in color, hard, and bound down to the diaphragm by numerous fibers; on section the structure of the organ could not be made out. The kidneys showed no abnormality. The adrenal glands were injected and swollen. On opening the small intestine it was found to contain a yellowish mucous material with a sweetish odor. Erosion of the mucosa was noticed to be limited to those areas that were deeply injected; no nodules seen in the mucosa anywhere.

*Thoracic cavity.*—There was present a slight excess of pleural fluid, altho there was no pleuritis noticeable. Left lung collapsed; color reddish, with numerous



hemorrhagic areas scattered over its surface; the right lung was not collapsed, was of a clay color, and showed many hemorrhagic areas at the root.

*Heart*.—There was no pericarditis present nor hemorrhages; a slight amount of cloudy fluid was present. Heart muscle appeared normal; blood within the organ was laky. There were no lesions of the valves.

**MICROSCOPIC.** **Lamb 1.**—Alcohol preservation; hematoxylin and eosin.

*Heart*.—Certain of the fiber bundles show parenchymatous degeneration together with some fatty changes.

*Lung*.—With the exception of a few scattered air cells the tissue is normal.

*Kidney*.—Tissue not well preserved, appearances doubtful; perhaps there had been parenchymatous degeneration.

*Small intestine*.—Normal.

*Liver*.—Parenchymatous degeneration with fatty changes is seen throughout the whole specimen, but of a mild order, and chiefly confined to the outer and middle zones of the lobules. No hyperemia.

*Lymph gland*.—Apparently normal.

**Lamb 2.**—Alcohol; hematoxylin and eosin.

*Heart*.—Normal.

*Lung*.—Hyperemia; two small areas of polymorphonuclear leukocytic infiltration in the immediate vicinity of two bronchioles; other places normal.

*Kidney*.—Acute diffuse glomerulonephritis; some fatty changes found in some of the tubules. No interstitial changes.

*Liver*.—The section shows a generalized cloudy swelling of a moderate degree, occasionally some of the lobules are more severely affected than are others. In one or two places there are accumulations of small round cells at focal points. The blood capillaries and smaller bile ducts are much compressed; central veins widely distended with blood. No cholangitis.

*Lymph gland*.—Appears normal.

*Spleen*.—Compared with the human spleen there is more connective tissue distributed throughout it than normal; otherwise there is nothing abnormal.

*Intestines*.—Small. Not sufficiently well-preserved for description. Large. Hyperemic only.

*Adrenal gland*.—Normal.

*Spinal cord*.—Too poorly preserved for description.

**c) Horse.** Just outside of Carlsbad an opportunity occurred of making an examination of the carcase of a horse said to have died of "alkali." As the animal had been dead for at least 15 hours and left exposed to a hot sun the body had been so invaded by *B. welchii* that the tissues were everywhere so heavily stained by laked blood that it was impossible to make any interpretations of a satisfactory nature.

However, near Otis, N. M., we had a chance to make an autopsy on a horse one hour after death, and in general the conditions found were closely similar to those seen in cattle.

**MACROSCOPIC.**—*Pericardium* contained a large quantity of clear, straw-colored fluid. There were no evidences of inflammatory reaction seen, and no ecchymotic spots. *Heart* exhibited beneath the epicardium of the right ventricle, around the base of the organ, and along the course of the coronary vessels a large number of ecchymoses of small but variable size. The myocardium, particularly of the right ventricle,



looked paler than normal; and on section small scattered areas of probable fatty change were seen. No valvular lesions existed.

*Lungs*.—The only appearances of note were great hyperemia and edema in all portions of the organs. There were no signs of inflammation to be noted anywhere.

*Liver*.—Enlarged (?), purplish red in color; capsule smooth, thin, and free of ecchymoses; friable; on section the lobules could not be made out on account of cloudy swelling.

*Spleen*.—Capsule thick, grayish in color, wrinkled, and exhibiting a few small ecchymotic spots. On section, the color was a chocolate red; soft; and the Malpighian bodies invisible. The organ was not seemingly enlarged.

*Kidneys*.—The kidneys appeared to show cloudy swelling, but as they contained much blood this was not readily and certainly established.

*Intestines*.—In the small intestines there were found at irregular intervals nodules lying in some instances close below the peritoneal surface and in others somewhat more deeply imbedded in the wall of the gut. Viewed from the outside they measured in diameter about 4 or 5 mm. and were elevated above the surface in some cases full 2 mm., in others they projected about 1 mm. Those in certain instances were of a gray-yellow color surrounded by a zone of injected vessels and one or two of the nodules showed some traces of localized peritonitis capping, as it were, the apex of the nodule. In all there were eight of these nodules. On opening the gut it was found that these nodules had their origin in an inflammation of some solitary lymph nodes situated deep in the mucosa. In one part of the small intestine close to a nodule was seen imbedded fairly firmly a piece of hard foreign matter, which was supposed to be a thorn that had been swallowed and had penetrated the intestinal wall; this was later on found by microscopic examination to be the necrotic core of one of the nodules that evidently had ruptured through into the lumen of the gut.

Some small and large patches of ecchymoses were found in the mucous membrane of the duodenum, jejunum, and ileum. The contents of the tract as a whole were largely made up of a greenish yellow, tenacious mucus. The colon appeared to be normal. The peritoneal surface of the abdominal cavity presented a few ecchymotic spots. There was a very definite odor of acetone on opening up the cavities.

MICROSCOPIC.—Formalin preservation; hematoxylin and eosin.

*Heart*.—Left ventricle: Altho striation can be generally discerned, the muscle fibers in many places are finely granulated, nuclei absent or very vesicular and staining poorly.

*Kidney*.—Some glomerulitis present. Acute parenchymatous nephritis of high degree present nearly everywhere. Hyperemia.

*Liver*.—More or less generalized parenchymatous degeneration throughout the preparation, affecting most intensely the middle and inner zones of the lobules, with accompanying fatty changes confined largely to the middle zone. Pigment of a brownish color is to be noted in the columns of cells in the more intensely degenerated zones. Considerable hyperemia exists throughout the section. No cholangitis.

*Intestines*.—a) Duodenum: Marked inflammation of the mucosa and submucosa as shown by the hyperemia, great round-cell infiltration, loss of columnar epithelium on the villi, and the swollen or atrophic conditions of the latter in several situations.

b) Jejunum: Conditions are somewhat similar to those in the duodenum only of a much less severe degree.

c) Ileum (section through a node in the wall of the gut): The preparation shows that the whole of the tissue is in a state of acute inflammation, there being intense

cellular invasion by round cells, polynuclear and large wandering cells, accompanied by marked edema from mucosa to peritoneum and hyperemia. The epithelium is lost from the major portions of the villi, and in one locality an ulcerative process has been in progress so that there has resulted a considerable loss of the several structures constituting the mucosa, so that there remains only a thin layer of mucosa above the muscularis mucosae but entirely devoid of any suggestion of epithelial structure. This zone of denudation is seen to be surrounding a conical mass of necrotic tissue whose base lies free toward the lumen of the gut, its tapering apical part passes outward through all structures of the intestinal wall until it rests upon the inner surface of the inner muscular coat of the bowel. Throughout its course this necrotic mass is sharply demarcated and partially loosened from the surrounding inflamed tissue. So far as can be ascertained its origin cannot be traced to a thrombosed artery as suggested by the shape of the mass. The subperitoneal tissue is very edematous and filled with various types of inflammatory cells, and altho in places the cells of the peritoneum itself are missing, it is in all likelihood due to rough handling of the preparation, as there is no coagulated lymph anywhere to be seen in the denuded areas.

**d) Human Case. AUTOPSY.**—We have seen but one fatal human case. This was one of the Morris cases elsewhere referred to (p. 416). Death occurred at 12 noon, November 26; the autopsy was begun at seven o'clock the same evening. (Oil light.)

**H. J., 8 years.**—The body is that of a white female child, 52 inches in length, and about 65 lbs. in weight; fairly well nourished. Trunk not yet cold.

Livor mortis discerned on left side of face and over all dependent parts. Rigor mortis of lower limbs; upper supple (undertaker present stated that the arms were also rigid, but had been broken down by him).

Mucous discharge from right nostril (stomach contents?); fecal discharge of a watery nature from the anus. Strong odor of acetone from the body.

On reflection of the skin the subcutaneous fat was found to be rather scant in amount. Pectoral muscles were dark red in color. The acetone odor was quite marked over the area denuded of integument.

*Abdominal cavity.*—Peritoneal cavity shows no excess of fluid and no signs of peritonitis; no ecchymoses visible; omental fat scant. The liver does not project below the costal margin; it is, however, apparently very fatty. The intestines (ileum) are empty and of a generalized pinkish color; the colon is filled with fluid contents and bluish-gray in tint. The stomach is partially filled with fluid; it is of a normal color tint. Pancreas firm and of a pale pinkish color. The spleen is not visible as the intestines lie undisturbed.

*Individual examination of the abdominal organs. The small intestine.*—The duodenum appears from the outside to be injected and this injection passes down for a short distance over the jejunum and then the rest of the gut is generally of a pink color. The mucosa of the duodenum was generally injected and somewhat edematous. On opening the jejunum it is found to contain a slight quantity of a tenacious yellowish mucus and the mucosa is of a definite pinkish tint everywhere; there does not seem to be any edema or inflammation, and there are no erosions of the mucosa. In the lower portion of the small intestine, the ileum, there can be seen before the gut is opened several dark purplish spots; these are found to correspond to the lymphoid plaques, Peyer's patches; in the opened gut they are found to be in a state of very moderate hyperplasia and are of a rather purplish-red color; in no instance do they show any signs of erosion, nor anywhere stand markedly above the general surface of the mucous membrane.

*The colon.*—The contents of this portion of the tract are brownish, watery, and have a fecal odor. In the descending portion there can be seen three or four small areas of congested vessels, otherwise the gut is pale in color. When washed free of contents and critically examined there is seen to be general folliculitis of mild degree; even when examined with a hand lens there cannot be discerned any process of erosion, simply hyperplasia.

*Stomach.*—Contents brownish watery; mucosa covered with sticky mucus; no rugae visible; seemingly normal.

*The mesenteric glands* are everywhere moderately enlarged and, on the whole, firm to the touch.

*The liver.*—The organ is of a markedly yellow color; not enlarged; capsule smooth and showing no ecchymoses; it is firm to the touch and is not friable. The gall-bladder is not distended and contains a dark brownish-green bile which is rather watery in consistence. On section, the general architecture of the organ can be made out without difficulty; the central parts of the lobules are brown in color while the peripheries are very distinctly yellow. No necroses of a focal nature were discoverable. The bile ducts and canals were nowhere distended.

*The kidneys.*—Both looked alike, if anything a trifle smaller than normal. In color they were of a yellowish-pink cast. Capsules everywhere smooth and non-adherent. No infarcts seen. On section, the cut surfaces had a very definite yellowish color, evidently fatty; the cortex was somewhat greater than the normal in depth; the interlobular vessels were readily discernible, but not so the Malpighian tufts (probably on account of the kerosene light); the pyramids were also of a fatty appearance. The pelves of the organs appeared normal. No chronic interstitial nephritis.

*The adrenal glands.*—Both seemed to be normal.

*The spleen.*—This organ did not appear to be enlarged. It was firm as is the normal. Color, a normal brown-red. Capsule not thickened. On section, the spleen did not contain any excess of blood; no interstitial splenitis; the Malpighian bodies stood out plainly; there were no infarcts found.

*The urinary bladder.*—It contained about 50 c.c. of a clear pale yellow urine (preserved). There were no signs of cystitis. The ureters appeared normal.

*Uterus and ovaries.*—The sex organs appeared normal.

*Pancreas.*—It seemed normal.

*Thorax.*—The pectoral and intercostal muscles were of a dark red color. On removal of the sternum the thymus gland was seen to be of a diamond shape measuring  $1 \times 1\frac{3}{4}$  inches  $\times \frac{1}{4}$  inch deep; it was soft to the feel of the fingers and did not appear to be abnormal in any respect. The precordial space measured about  $2\frac{1}{2} \times 2\frac{1}{2} \times 3\frac{1}{2}$  inches.

*Pericardium and heart.*—The pericardial sac on being opened was seen to contain about 15 c.c. of a clear yellow fluid (some put in a sterile tube). There were no ecchymoses on the parietal walls. No signs of pericarditis. The heart did not seem to be enlarged; the cavities of the right side were filled with blood, while the left chambers appeared to be empty. Beneath the visceral pericardium at the base of the heart and at the roots of the pulmonary artery and aorta were seen a number of very small ecchymotic spots not measuring more than 1–2 mm. in diameter; a few were also found alongside the veins of the anterior interventricular groove; these were quite minute. On opening the heart the right auricle and likewise the right ventricle were found to be filled with postmortem clots; the foramen ovale was closed and the endocardium appeared to be normal. The right ventricle: The tricuspid valves were found in a normal

state, as also the pulmonary-artery valves; the endocardium everywhere was smooth and showed no ecchymoses. The left side: There was nothing found of an abnormal nature in the left auricle or in the left ventricle. The heart muscle generally seemed to be of normal consistence and on section appeared to be of a good color and gave no evidence of myocardial changes.

*The lungs.*—These organs did not collapse greatly on the opening of the thorax. The pleural surfaces were everywhere smooth and showed no visible signs of inflammation. There was no excess of fluid.

The *left* lung was voluminous; along the free edges the upper lobe was a little emphysematous and pale red in color; elsewhere the organ was of a dark red color, crepitant, but boggy. On section, the upper lobe gave evidence of chronic passive congestion, while the lower lobe was very edematous, considerable quantities of frothy fluid being set free on pressure; the bronchi did not appear to be inflamed.

In the *right* lung, as in the left, the upper and the middle lobes showed evidences of chronic passive congestion; the lower lobe in greater part was of so dark red a color and so airless as to look like liver; it was hepatized, but in small portions toward the anterior it contained some air and was crepitant. The bronchi in the middle and the lower lobes in some places were in a state of inflammation and pus oozed out of them on being cut across.

*Brain and meninges.*—The calvarium was removed and the meninges and the brain examined carefully; so far as could be seen there were present no abnormalities. Veins on cortex somewhat turgescient.

MICROSCOPIC. H. J., Morris, Ill.—Tissues in formalin; hematoxylin and eosin.

*Heart.*—Right ventricle: In cross-section of a part of the specimen there are found not a few fibers that contain fat droplets, centrally disposed. In longitudinal section, loss of striation is the only feature to be noted departing from the normal. Left ventricle: In cross-section one can detect areas in which are the cut ends of certain bundles broken up in granular fashion; nuclei are less abundant, and fat droplets can be detected here and there. In longitudinal section no bundles appear in the preparation suggestive of myocardial change; striation and other conditions of the muscle cells appear to be normal. There is, however, a definite degree of interstitial inflammation manifested in the increase in number of the nuclei of the connective tissue lying between the muscle bundles together with some round- and polynuclear-cell invasion.

*Cerebrum and cerebellum.*—Apparently normal. Vessels of the pia-arachnoid hyperemic.

*Lung.*—Much hyperemia; air-containing alveoli numerous, but in other places there is edema or atelectasis. No signs of any kind of inflammatory reaction.

*Kidney.*—Acute diffuse parenchymatous nephritis and glomerulitis. In company with the parenchymatous changes there is a high grade of fatty metamorphosis in many of the tubules, the fat being laid down in the shape of fine droplets.

*Liver.*—The cells of the parenchyma present a high degree of fatty degeneration everywhere, the cells being loaded down with small and large fat droplets. The nuclei of the cells are often absent or stain poorly. The blood channels are very hard to see on account of the generally swollen condition of the parenchymal cells, while the smaller bile ducts are compressed and the central veins widely open and filled with blood. There is one minute focal point of round-cell invasion, not accompanied by any signs of necrosis. No focal necrosis; no cholangitis.

*Intestines.*—All specimens show lack of sufficient penetration of the preservative

to allow of any trustworthy interpretation of appearances. If one may judge from what is presented hyperemia only prevails.

*Thymus gland.*—Apparently normal.

*Small intestine.*—Zenker preservation; hematoxylin and eosin. Section through a Peyer's patch. There are the usual signs of inflammatory reaction in the close neighborhood of and in the lymphatic tissue of the patch, there being loss of columnar epithelium, cellular invasion of the villi, and engorgement of the veins. The Peyer's patch shows inflammatory hyperplasia but no necrosis of its substance anywhere.

In addition to this case we have obtained through the courtesy of physicians material from two other human cases.

**Dr. Goldsmith's Case.**—This was material from one of the fatal cases in New Mexico mentioned on p. 416 (M. H.). It was kindly given to us by Dr. A. A. Goldsmith, of Chicago, to whom it had been sent for examination in January, 1907. Formalin preservation; hematoxylin and eosin.

*Lung.*—Considerable general hyperemia. Many alveoli edematous and in places the red cells have escaped as well into the alveoli. There are no evidences of bronchitis, bronchopneumonia, or pneumonia.

*Kidney.*—Intense diffuse parenchymatous and glomerular nephritis. Some necrosis and much fatty degeneration of the convoluted tubules, as well as fatty changes in the descending loops of Henle and of the collecting tubules. The tissue was probably hyperemic, has been affected by its prolonged stay in the formalin so that the blood cells are not particularly well preserved.

*Liver.*—If the bile ducts and the blood vessels of Glisson's capsule could not be made out, the section would be doubtless taken for adipose tissue, so intense is the fatty degeneration present. The nuclei of the liver cells can be made out only here and there.

*Spleen.*—Some evidence of postmortem change throughout the specimen. In all likelihood the organ was considerably hyperemic, the size of the Malpighian bodies being encroached upon. There might have been some swelling of the cells of the reticulum together with some increase. No increase in the connective tissue apparent.

**Dr. Maple's Case.**—This case occurred in Shelburne, Ind., in the fall of 1906, and we are indebted to Dr. James B. Maple, of Shelburne, and to Dr. B. V. Caffee and Dr. Millard Knowlton, of Terre Haute, for information concerning it. The autopsy was made by Dr. Maple who has kindly furnished us the following notes:

Boy, James, age 16 years; previous health good; mother died a few days earlier of the same disease. Had been sick three weeks and was suffering from relapse when seen; sweetish breath, frequent and severe vomiting, bowels had not moved for 15 days, temperature was subnormal until day of death when it rose to 102°. Autopsy findings: *Thoracic cavity:* Lungs edematous, heart negative. *Abdominal cavity:* Not distended, no peritonitis, visceral peritoneum injected. *Stomach:* Contained nothing except about a pint of castor oil mixt with a greenish fluid; contents had a sweetish smell, like that of the breath; the mucosa of the stomach was highly inflamed and contained one ulcer with another area which was just ready to ulcerate; there was evidently an intense gastritis. *Intestines:* Both large and small were distended to their usual size with gas; on slitting them from one end to the other less than a gill of contents was found; there were constrictures every few inches in the course of the small gut, but there did not seem to be any scar tissue at these places. *Mesenteric lymph nodes:* Were enlarged and seemed to be inflamed. *Liver:* Was much enlarged, yellowish-red, and hard "as if



it were stuffed with fat." *Pancreas*: Size normal. *Spleen*: Seemed normal. *Kidneys*: Were enlarged, probably one-third. *Brain*: Was not examined.

**MICROSCOPIC.**—Specimens of the tissue sent us by Dr. Knowlton gave the following findings: Formalin preservation: hematoxylin and eosin.

*Heart*.—Left ventricle: Cross-section shows much apparent fatty change as seen in the ends of many of the fibers of certain bundles; bundles somewhat widely separated. In longitudinal section striation is everywhere plainly marked and the fibers appear normal. There may be some edema present as the fibers are at times widely apart and the connective-tissue cells stretched from side to side as if by pressure.

*Lung*.—Many small areas of atelectasis; hyperemia; no general edema; no inflammatory reaction anywhere.

*Kidney*.—Intense diffuse parenchymatous nephritis and well-marked glomerulitis. Considerable fatty changes in the affected tubules pretty generally. No interstitial nephritis.

*Liver*.—Organ so fatty as to be barely recognizable; nuclei of cells greatly diminished in number. Blood channels barely discernible.

*Spleen*.—Hyperemic; on this account some of the Malpighian bodies are much lessened in size and less well defined.

*Pancreas*.—Apparently normal.

*Intestines*.—a) Small: Solitary follicles are markedly hyperplastic with invasion of small round cells into the neighboring mucosa accompanied by necrosis of epithelium of glandular structure. Much hyperemia.

b) Large: Considerable hyperemia; in two small foci are evidences of a mild degree of round-cell infiltration, with epithelial desquamation, mucoid degeneration of epithelial cells, and edema.

#### THE CAUSE OF MILKSICKNESS.

**a) Mineral Theory.**—One of the earliest views regarding causation was that some poisonous metallic substance was present in the soil of the affected region and that this was taken up by the vegetation, and caused poisoning in grazing animals. This was the view held by observers in different localities; for example, in Alabama (Shelton, 1836), Indiana (White, 1836), and Kentucky (McAnelly, 1836). One of these writers (McAnelly), who believed that the poison was contained in water as well as soil, would account for the autumnal incidence of the disease by the explanation that the water in wells is usually at its lowest level in the fall and consequently the poison is "more concentrated."

Arsenic and copper were the two substances most commonly accused of being the responsible elements. Seaton (1842) held strongly to the arsenical hypothesis, but his argument was neatly summarized by Drake (1842) in the following words: "Cattle die of trembles—a cow died from taking arsenic, *ergo* the trembles are

produced by arsenic," a mode of statement not inapplicable to the conclusion drawn by some other experimenters. Strong ground against the mineral theory is taken by Yandell (1852) who asks: "Who can believe that the flesh of cows may be so saturated with arsenic or copper or the salts of mercury as to kill hogs, dogs, and buzzards?" None of the writers appear to have made systematic tests for the presence of poisonous minerals in soil or in the bodies of dead animals.

The facts of geographical distribution do not favor the hypothesis of a dependence of the disease upon a particular constituent of certain soils. Milksickness occurs both in the glaciated region of Ohio, Indiana, and Illinois, and in the non-glaciated states of Kentucky and Tennessee. We have compared carefully the areas of past and present prevalence of the disease with geological maps of the affected regions and are able to state positively that there is no connection between the geological and chemical character of the underlying rock and the geographical distribution of the disease. In the state of Kentucky, to take only a single instance from the mass of data that we have examined, there are records of the occurrence of milksickness on the Orthovician Formation (Boone and Campbell counties), the Carboniferous (Monroe and Todd counties), the Silurian (Henderson and Davis counties), and the Quaternary (Graves County).

A new turn has been recently given to the theory of mineral poisons by Moseley (1908) who has fed rabbits with aluminum and magnesium nitrate and also with *Eupatorium ageratoides*, the ash of which contains these salts.<sup>1</sup> According to this writer some of the symptoms of trembles were produced in this way.<sup>2</sup> The experiments and their explanation were seemingly suggested by the observations of Crawford (1908) on barium as a cause of loco-weed disease. Full details of clinical symptoms and pathological change have not yet been published. We have not ourselves attempted to repeat these experiments with mineral substances, but we have made thorough search for poisonous ingredients in the organs of one fatal human case of milksickness. No arsenic, copper, mercury, or barium was

<sup>1</sup> Both Mg and Al appear, however, to be not uncommon constituents of plant ash (Czapek, *Biochemie der Pflanzen*, 2, pp. 796-99).

<sup>2</sup> When aluminum and magnesium salts are taken into the human alimentary tract, commonly no symptoms of poisoning are observed (Cushny, *Pharmacology and Therapeutics*, 1903, 3d ed.).

found in the stomach contents or spleen. A complete mineral analysis of the liver showed only the following metals present: Fe, Ca, Mg, Na, and K. An analysis, including spectroscopic examination, of the incriminated butter (Morris case, p. 417) showed Na as the sole metal present; no trace of Al or Mg was found. Our observations, therefore, so far as they go, lend no support to the theory that trembles and milksickness are due to mineral poisoning.

**b) Poisonous-Plant Theory.**—The connection of the disease with particular areas and seasons, and perhaps also certain other epidemiological features, has caused numerous writers to adopt the theory that some poisonous plant eaten by cattle is the cause of trembles. There has been no universal agreement, however, as to what particular plant should be held responsible. The poison ivy (*Rhus toxicodendron*) and the white snakeroot (*Eupatorium ageratooides*) have been most commonly accused, but numerous other plants have been suspected. In certain localities and at various times the following plants have been held to cause trembles: *Lobelia inflata* (Indian tobacco), *Bignonia capreolata* (cross-vine), *Apocynum cannabinum* (Indian hemp or Indian barley), *Caltha palustris* (marsh-marigold), *Euphorbia esula* (spurge), *Aethusa cynapium* (fool's parsley), "*Vitis hederaceae*," now *Pseuderia quinquefolia* (Virginia creeper), "*Symphoria glomerata*," now *Symphoricarpos orbiculatus* (Indian currant), *Cicuta "virosa,"* probably *C. maculata* (cowbane). In the Pecos Valley, N. M., we found that the rayless goldenrod (*Bigelovia rusbyi*) was believed by some to be the cause of the "alkali poisoning" observed in cattle, altho at the same time we were told by many cattle-men that the disease was known among cattle grazing on certain spots where "nothing grew but salt-grass" (*Distichlis spicata*).

The poison-ivy theory at one time obtained wide currency, apparently largely through the publicity given it by Drake (1836 and 1841), and is often referred to as Drake's theory. This author, however, seems himself to have been somewhat lukewarm in his advocacy of it. He says (Drake, 1840), "that this plant (*Rhus toxicodendron*) is the cause of trembles . . . may be said to be the popular opinion of the district." Drake then declares that this opinion is "highly probable" altho he forthwith admits that "there is no conclusive evidence

of a single case of trembles having been produced by the *Rhus*." Among other writers expressing a more or less strong belief in the poison-ivy theory are Travis (1840), Cook (1857), Chase (1861), and Elder (1874). Barbee (1840) states that he had killed dogs with decoctions of *Rhus toxicodendron*, but makes a similar assertion about *Eupatorium ageratoides*.

Numerous writers oppose the theory that trembles is a *Rhus* intoxication. Some, as Henry (1854) and Beach (1883), point out that horses, cattle, and sheep frequently eat the tender foliage of this plant with relish and evince no signs of injury, while others, as Landrum (1861) and H. M. K. (1862), call attention to the entire absence of milksickness in numerous localities where the poison ivy grows in profusion. The supposed relationship of white snakeroot (*Eupatorium ageratoides*) to milksickness has been recently discussed by Crawford (1908) and need not be fully considered here. Barbee (1840), Dewey (1856),<sup>1</sup> Jerry (1867), Townshend (1883), and Moseley (1906) recount feeding experiments which in their opinions establish etiological relations between *Eupatorium* and milksickness, but Beach (1883) and many others state that cattle frequently eat this weed without any obvious ill effect. The white snakeroot does not grow in New Mexico where we observed typical trembles in cattle. Crawford's painstaking experiments upon various susceptible animals and upon himself afforded no evidence that the disease is due to any constituent of *Eupatorium ageratoides*. Moseley's recent modification of the *Eupatorium* theory is referred to above.

Other plants are mentioned as the possible or probable cause of the disease by McCall (1822), Short (1840), Fisher and Kennicott (1861), and Allen (1878), but the evidence adduced is, if anything, more inconclusive than that already cited in the cases of *Rhus* and *Eupatorium*. Coleman (1822) experimented in regard to the poisonous qualities of certain plants, but was unable to find any that would produce the peculiar symptoms of the disease. Crookshank (1826) argues strongly against the poisonous-herb theory, but J. N. Smith (1837) and McNutt (1847), on the other hand, agree with the view

<sup>1</sup> This writer's observations apparently refer to the white snakeroot, but he did not take the pains to identify the plant upon which his observations were made, altho he adds remorsefully, "I intended to have learned to what class in botany this plant belongs before this meeting, . . . but have been prevented by procrastination."

that the poison is of a vegetable nature. Winans (1840) and Johnson (1866) are of opinion that the poisonous agent is the mushroom. Slack (1854) and Nagle (1859) believe the cause to be a "fungus production" on grass or grass seeds, the first-named author comparing milksickness to ergot poisoning. It is worth noting that in some localities cattle and horses affected with ergot poisoning are said to be "alkalied."<sup>1, 2</sup>

c) **Microbic Theory.**—Many of the earliest observers felt dissatisfied with the explanations of the causation of milksickness current at the time they wrote. This feeling is manifested by the promulgation of various fantastic hypotheses, some of which have been cited, and especially by the frequent comparison of milksickness to malaria and the ascription of the former to a "miasmatic origin." Wright (1877) after a discussion of the subject concludes "that the cause of the sick stomach is still somewhat doubtful, and will remain so, so long as it is sought for amongst the vegetable or mineral poisons." Reed (1866), after recounting the failure of chemical analyses to show the presence of a mineral poison and of botanical researches to reveal the existence of a specific poisonous plant, remarks, "What success may be had in searching for a cryptogamic or animalcular cause remains to be shown." De Bruler (1858) is forced to believe

<sup>1</sup> *Breeders' Gazette*, September 25, 1907, p. 572.

<sup>2</sup> *Miscellaneous theories.*—Hardly any remotely plausible hypothesis of the cause of milksickness is without advocates. Many writers claim that a strong resemblance exists between this disease and malaria, (Lescher, 1850; Thompson, 1853; Sale, 1878; Rawlings, 1874; Dorsey, 1876). Lea (1821) express his belief that the disease was due to a "miasmatic exhalation." Horne (1846) believed that the "exciting cause" of milksickness and yellow fever is the same and that "the agent is miasmata." Wozencraft (1873) maintained that the disease was due to "an exhalation from the soil." Jones (1862) states his somewhat inclusive opinion as follows: "I conceive the cause of this disease to be a peculiar miasma, generated under peculiar circumstances. It may be an animal sporule; a vegetable atmospheric fungus; or a gas formed by a particular combination of the elements." The same writer adds: "We generally, I may say invariably, find milksickness in or near a notoriously miasmatic district."

Kennedy (1878) thinks that he detects a similarity between the disease and the tsetse-fly disease in Africa described by Livingstone, and accordingly attributes milksickness to the bite of a "certain fly." Achelor (1884) likewise ascribes the disease to the bite of "the milk sick fly," an insect he does not further differentiate. Carpender (1884) and Murfin (1884) vigorously attack Achelor's fly theory.

An anonymous writer (S., 1820) in the *Transylv. Jour. Med.* refers to experiments made about 1800 which gave rise to the conviction that the disease was occasioned by cattle eating "the mass of vegetable matter growing in the bottom of stagnant pools of water." Jackson (1881) attributes the disease to the "seeds of a vegetable." Thompson advances the view that milksickness may be due to an "excess of carbonic acid floating in the atmosphere," and so would account for the greater prevalence of the disease in dry seasons by the premature falling of the leaves (which remove CO<sub>2</sub> from the atmosphere). Another writer (S. M. B., 1892) has no hesitation in asserting that the disease is caused by swallowing the web of the spider. "It is an admitted fact that a spider web when fresh is poison, but a little sunlight and heat rob it of its virulence, and it becomes an inert absorbent." There seems no need to push historical inquiries any farther.



that the poison is not vegetable or mineral, and continues: "But there is a large class of animal poisons commonly called infections which do possess the power of self-propagation, whenever they are placed in a proper condition for such development." He cites hydrophobia as an illustration and adds: "Infectious diseases are sometimes the results of animalcules. May they not always be produced in the same way?" Woodfin (1878) is also inclined to believe that the disease has an animalcular origin. Philips (1877) who was evidently strongly under the influence of the germ theory of disease points out that milksickness is a specific infection, that it has an incubative period, is transmissible, and reproduces its kind. He reports observing in freshly drawn blood and in urine "a great number of living, moving, spiral bacteria." Logan (1881) express his belief that the disease is due to a "*contagium vivum*," without, however, giving any specific observations in support of his opinion. About the same time Gardner (1880) reports seeing "countless multitudes of actively moving, writhing, twisting bacteria" in the blood of a sick heifer and also in the blood of two human patients. A fungus (*Sterigmatocystis*) has been obtained by Hessler (1905) from the blood of a sick horse.

#### EVIDENCE IN FAVOR OF THE MICROBIC THEORY.

##### **Epidemiological Resemblance of Milksickness to Anthrax.—**

There is a close correspondence between the epidemiological features of milksickness and those of anthrax in cattle. As shown by Friedrich (1885) anthrax is primarily associated with infected pasture land and occurs most commonly in certain well-known localities. Its prevalence in particular regions (e. g., the Bavarian Alps) is dependent in the first instance on variations in soil moisture and is hence affected by seasonal differences in temperature and rainfall. Friedrich emphasizes the influence of drainage in diminishing the amount of anthrax. "Wenn es gelang, den Milzbrand aus einer Gegend zu vertreiben, oder doch zu reduciren, so ist dies nur der Trockenlegung der versumpften Milzbrandlocalitäten zu verdanken. So verschwand der Milzbrand nach Flussregulirung, oder er nahm ab, als man die gefährlichen Weiden in Ackerboden umwandelte." According to Bollinger (1885) in the Bavarian Alps anthrax is "eine exquisite Bodenkrankheit," and is most likely to occur when a wet year is

followed by a less wet or a dry year accompanied by high temperature. These statements, as shown in the foregoing sections of this paper, apply equally well to milksickness. The persistence of both milksickness and anthrax in given localities is dependent in some way upon the content of soil moisture. Bürger (1825) long ago drew attention to another analogy between milksickness and anthrax, both diseases, as he expresses it, being communicated through milk and meat. Recent writers and bibliographers generally accept the theory of a parasitic origin (Maurel, 1889; Boggs, 1907); Crawford, 1908; McCoy, 1907. Certain facts already set forth respecting the nature of the virus strongly support the microbic theory. Such are the destruction or weakening of the virus by heat, the apparent connection of the virus with particular tracts of soil while adjoining areas that are chemically and botanically similar are entirely free of it, and especially the self-propagating power of the virus which has been long recognized as one of its most characteristic properties.

Our own observations bear upon the microbic theory and may now be described in detail. The first cases of the disease coming under our notice were in New Mexico. Blood films, which were made from a human case (above, p. 456) and stained by the Romanowsky and other methods, did not show the presence of any microorganisms. Agar tubes inoculated with the blood from the elbow vein gave no growth. The case was quite far advanced in convalescence. A young bull apparently in the early stages of trembles was shot and an autopsy made while the body was still warm (rectal temperature 103.2°). Agar tubes inoculated with bile and with heart blood gave no growth. Rabbits inoculated with serum (intraperitoneally, 1.5 c.c.) and with bile (subcutaneously, 2 c.c.) remained well.

Following these preliminary experiments we had the opportunity of making a more thorough examination of the body of a young pregnant heifer five hours after death. Coverslip films were made from spleen, liver, kidney, heart blood, lung, and gall-bladder, and from the liver of the fetus (four months?). In the films from all the organs a long slender bacillus, staining uniformly with methylene blue, was found in very small numbers (1 to 8 per film). No bacteria were found in the films made from the heart blood. Plate cultures

were made on agar with heart blood, liver, spleen, kidney, and fetal liver; two tubes of slant agar were inoculated with two or three loopfuls of bile removed from the gall-bladder after careful searing. After 12 hours' incubation at 32° C. the agar tubes showed profuse growth. Films stained with methylene blue showed apparently pure culture of a bacillus with deeply stained metachromatic granules and round terminal spores.

On the agar plates, after 36 hours' incubation at 32°–36° C. a few small irregular, transparent colonies appeared. There was an average of about six colonies to a plate; the plate from the spleen showed a mixture of colonies; the others were apparently a pure culture. Bacilli from several colonies were stained and examined microscopically and were morphologically identical with those found in the bile cultures. Pure cultures were eventually obtained from the plates made from the liver and fetal liver and they were in every respect identical with the microorganism found in the bile. A young rabbit (about 400 gm.) was inoculated intraperitoneally with 2 c.c. of heart blood from this heifer; the rabbit died in four days and the same bacillus discovered in the bile and in the fetal liver of the heifer was found in pure culture and in large quantities in the peritoneal exudate. Two rabbits inoculated, respectively, with the peritoneal

#### PRESENCE OF *B. LACTIMORBI*.

Animal	Organ or secretion	Animal	Organ or secretion
Heifer P <sub>1</sub> . . . . .	liver	Lamb <sub>1</sub> . . . . .	brain
" . . . . .	bile	Lamb <sub>2</sub> . . . . .	heart blood, liver, and
" . . . . .	spleen		peritoneal fluid
Fetus . . . . .	liver	Child H. J. . . . .	spleen
Heifer P <sub>2</sub> . . . . .	bile	" . . . . .	duodenal contents
Steer P <sub>1</sub> . . . . .	intestinal contents	" . . . . .	pericardial fluid
	(mucus)	Man M. . . . .	feces
Heifer P <sub>4</sub> . . . . .	intestinal contents	Man H. . . . .	"
	(mucus)	Man A. . . . .	"
Horse <sub>1</sub> . . . . .	gut nodule	Cow R. . . . .	milk
" . . . . .	pericardial fluid	Cow P. . . . .	"

exudate (0.75 c.c.) and heart blood (1.5 c.c.) of this rabbit also died. The bacillus thus discovered in the organ of a fatal case of "alkali poisoning" or trembles has since been found by us in every case of the disease that has come under our observation. The preceeding list gives our findings in detail. In several instances the bacillus was

present in pure culture, namely Heifer P<sub>1</sub>, bile, and fetal liver, Heifer P<sub>1</sub>, bile, Horse<sub>1</sub> gut nodule, intestinal wall and pericardial fluid. In other cases it was accompanied by *B. coli* or rarely by staphylococci. We have designated this bacillus as *B. lactimorbi*. As we have elsewhere (Jordan and Harris, 1908) described the character of the bacillus with some fulness we will simply quote the essential points of that description here.

#### DESCRIPTION OF BACILLUS.

The morphologic and tinctorial features of this specific organism may be briefly given as follows: In coverslip smears from the organs the bacilli are longer and more slender than the colon bacilli, and stain occasionally unevenly with methylene blue. (Fig. 5.)<sup>1</sup> In preparations made from cultures grown on agar at 37° C. the organism



FIG. 5.—*B. lactimorbi*. Smear, peritoneal cavity, Rabbit 5. Methylene blue.  $\times 1100$ .

is found to be a rod a little smaller than the anthrax bacillus, occurring singly and in pairs and in occasional filaments. As a rule the rods at the end of 24 hours' incubation do not stain deeply with methylene blue, even if heated for a moment or two over the flame, but at one or both poles or at the center of each rod are found metachromatic granules which take on a reddish or purple tint, standing out sharply from the rest of the stained rod. In young culture the bacilli are gram-positive but the granules are not differentiated by the gram stain. Spore-formation was observed in our first culture, the spore being perfectly round and situated at one pole of the rod in the mature forms; in the less mature rods, 24 hours old, the spore may be slightly oval and lie not quite at the end of the rod; the general effect produced by the shape and location of the spore reminds one strongly of the tetanus bacillus (Fig. 6). Rarely one rod may show bipolar spores.

<sup>1</sup> For the preparation of the photomicrographs we are under obligation to Mr. A. C. Hicks, Assistant in Bacteriology.

The spores stain readily by any of the ordinary methods. The organism is motile and it is found on staining by van Ermengem's method to be possessed of 10 or 15 flagella disposed peritrichally, the flagella measuring in length about five times the length of the bacillus itself.

This bacillus grew on our plates as small streptococcus-like colonies. It was soon found that some of the bacilli isolated and possessed of similar cultural characteristics did not answer in all respects to the description just given. Some differed markedly in morphology, forming more filaments than rods, both being almost devoid of metachromatic granules and in some cases even of spores, although corresponding culturally. This morphologic deviation occasioned some perplexity until it was found that if grown on gelatin at 25° or 30° C. the refractory bacilli would become metachromatic and sporogenous. We were able at the outset to establish the presence of *B. lactimorbi*

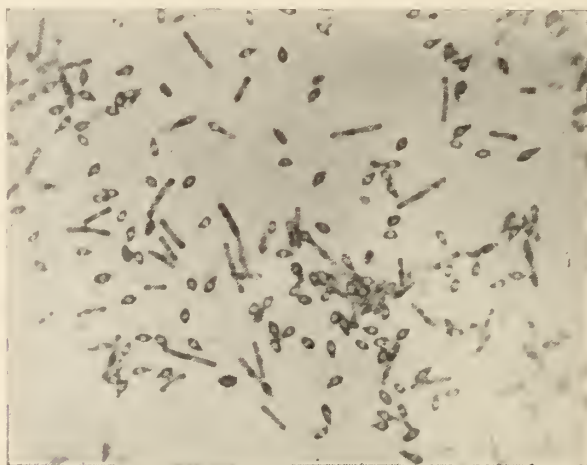


FIG. 6.—*B. lactimorbi*. Pure culture from P. H. 1, bile. Six-day growth in Jordan's asparagin solution. Methylene blue.  $\times 1100$ .

in the cultures from four animals from the following sources: In the intestinal contents of two cows, in the bile, spleen, and liver, and in the liver of a four months' fetus of another (intestinal contents not examined), and in the pericardial fluid and intestinal nodule of a horse.

Studied in greater detail after we had become assured from the results of animal inoculation that we were in possession of a pathogenic microorganism, the following appearances and cultural reactions were observed:

*Agar slant*.—At 37° C. at the end of 24 hours, the surface is more or less irregularly covered with a delicate veil-like growth, which is more profuse at the end of from 48 to 72 hours and eventually may take on a semiviscid character in some cultures; color, grayish, moist, smooth, and glossy; no pigmentation of the growth itself or of the medium; no gas; condensation-water growth heavy, gray-white in color. No odor.

*Dextrose-agar stab*.—Surface growth occurs around the puncture and extends irregularly around from it in a delicate manner. At first the bacterium grew sparingly



down the middle track for about 1.5 cm. but now it grows down 3 or 4 cm. or even farther; the needle growth is smooth and delicate, whitish in color and smooth; no pigmentation of the medium; no gas; no odor. The growth is more luxuriant after a few days.

*Broth.*—At the end of 24 hours there may be not any growth noticeable except sometimes a slight clouding of the broth at the surface. At the end of 48 hours there is seen on the surface, a well-formed pellicle, which will sink if the tube is agitated; the rest of the medium is feebly clouded and there may be a fine semiflocculent sedimented growth at the bottom of the tube or along the dependent side, if the tube has been inclined.

*Litmus milk.*—If not heavily seeded there may be no visible signs of growth. As a rule there is no change at the end of 48 hours; by 72 hours the cream ring will show a delicate green-blue color in the drier parts, and even in some instances the upper portion of the milk will show a slight alkaline reaction; this reaction daily increases until at the end of ten days the whole tube takes on an alkaline reaction which becomes more and more marked until as in cultures of the hog-cholera bacillus the medium may lose a part of its color, turning a dirty white, and even begin to turn opalescent, indicating a solution of the casein due to the great increase in free alkali. Coagulation has not been observed.

*Potato.*—No growth was observed; no multiplication was apparent on examining the surface by scraping and staining the scrapings and examining them under the microscope.

*Heinemann's synthetic potato medium.*—No visible growth could be made out even at the end of two weeks; microscopic examination of the scrapings of the medium at the end of two or three days shows evidence of abundant multiplication.

*Blood serum* (Löffler's).—At the end of 48 hours there occurs along the needle track and confined to it an elevated, fairly vigorous, smooth, moist, yellowish, glossy growth, which later tends to spread but little. There is no discoloration of the medium, nor any liquefaction, nor odor.

*Gelatin stab.*—Grown at 20° C. growth begins to appear at the end of 48 hours, but is not sufficiently advanced for description until 72 hours old; then there is seen little or no surface growth around the puncture; down the stab to the extent of about 2 cm. a delicate gray, smooth growth appears. At the end of a week the surface growth has become extended on all sides from the puncture as a fairly well developed film of a whitish color, smooth, moist, and glossy; at times the first evidences of liquefaction may now be detected, but this is not well established until the tenth day, when the medium just beneath the surface growth is slightly fluid. As time passes the liquefaction slowly progresses, extending down the stab to a slight degree, the gelatin at the end of 18 days or three weeks being liquefied completely across the tube and slightly downward. If kept at 23° or 24° C. the appearance and progress of the liquefaction makes more rapid headway, and at the end of a month at this temperature the whole of the gelatin may be reduced to a fluid state. There is always noticeable a more or less well defined putrescent odor.

*Gelatin.*—If the organism is grown in a petri dish of gelatin at 30° C., it is found at the end of 24 hours that the whole of the medium is quite cloudy, with not infrequently several small islets of pellicular material scattered over the surface. The surface-growth increases during the next four days but never forms a complete film over

the surface. The putrescent odor is more manifest in cultures grown at this temperature (30° C.).

In all cases in which a surface film forms at room temperatures on any of the media, fluid or solid, and particularly in gelatin left at 30° C. spore-formation is abundant.

When grown at 37° C., particularly at the first isolation, the organism presents certain noteworthy differences both in growth appearance and in morphology. On slant agar the growth may continue thin and veil-like or may be as vigorous as the growth of the typhoid bacillus on the same medium. In broth there may or may not be a pellicle; as a rule at the end of 48 hours, there is a slight cloudiness only and a tolerable amount of sediment in the tube, which increases only slightly thereafter. The alkalization of the milk cultures is not hastened by growing at this temperature. The morphologic alterations are striking; the size of the bacillus is less, approaching that of the typhoid bacillus, and there are irregularities in staining both in the individual cells and in different cells in the field; filament-formation is more pronounced, and there may even be swollen, twisted forms of a shape entitling them to be classed as involution forms; metachromatic granules may be entirely absent or a few cells containing them may be found after prolonged search; spores are sometimes formed but as a rule not so abundantly as at 30° C. and by some races not at all. If a culture with these characteristics is grown on gelatin at 20° C. or at 30° C. and allowed to pass to the stage of liquefaction and again grown on the same medium and at the same temperature the type with metachromatic granules and abundant spore-formation is produced. Our recognition of the true nature of certain cultures was considerably retarded by the uncertainty introduced through this polymorphic tendency.

Agar-plate colonies of *B. lactimorbi* at the end of 24 hours at 30°, or 37° C., resemble those of *Strept. pyogenes*, but with this difference that they show a tendency to spread out in a film, particularly if the agar is freshly made, when the whole surface of the plate may be covered; film formation is also to be noted on the lower surface of the medium against the glass bottom. One of the strains at the end of three days on agar at room temperature developed a very distinct yellow color both in the depths of the agar and on the surface. In gelatin plates grown at 20° C. at the end of from 43 to 72 hours the colonies make their appearance and resemble at first those of streptococci but at the end of the fourth day they appear more vigorous, the surface colonies partaking more of the characteristics of *Staph. albus*, altho the color is of a yellowish-white and they are not so elevated nor large, measuring about 2 mm. in diameter. Liquefaction becomes apparent at the end of ten days or two weeks, the colonies settling down in the medium which after five or six weeks becomes almost wholly liquefied.

The thermal death-point of the bacillus as determined by the use of Sternberg's bulbs varies according to the type of culture employed, as shown thus:

48-hour agar culture grown at 30° C., fully sporing.....15' at 100° C.

48-hour agar culture grown at 37° C., non-sporing..... 5' at 55° C.

All tests were made with a time limit of 90 hours' incubation in the thermostat at 37° C., using agar as the medium for growth.

**Agglutination.**—Our first experiments upon agglutination gave negative results (Jordan and Harris, 1908), but later observations have shown that under some conditions agglutination occurs. Human serum from two of the Altamonte cases ("A" and "M") agglutinated

the bacilli from one of the other cases ("H") in 1:50 dilution (microscopic method) and also strains from the organs of a lamb (Morrow) and from Altamonte soil. One of the sera ("M") agglutinated *B. lactimorbi* obtained from the milk of one of the cows presumably concerned in the production of the Altamonte outbreak; the other serum ("A") had no effect upon it. Cultures from one of the New Mexico cattle, and from soil in Ohio were unaffected by either serum.

Another test with human serum ("H. J., Morris") gave a distinct agglutinative reaction upon two strains of *B. lactimorbi* (macroscopic method, 30 min. at 37.5°) in dilutions of 1:50 and 1:200, while the 1:500 dilution showed only a slight agglutination, hardly more than the trace of spontaneous flaking observed in one of the controls. This serum did not agglutinate *Bacilli typhosus*, *paratyphosus A*, and *enteritidis* Gärtner in dilution of 1 in 50.

**Distribution of *B. lactimorbi*.**—The striking and somewhat peculiar character of the microorganism we have isolated enables its presence to be rather easily detected and its identity quickly determined. Since we were not able to find in the literature any description of a bacillus possessed of the characters of *B. lactimorbi*, even in special studies of aerobic spore-bearing bacilli (Ford, 1901), we were at first led to believe that a peculiar and highly localized distribution of *B. lactimorbi* in the soil of certain districts might explain the singular geographical incidence of milksickness. This has not proved to be the case: we have found *B. lactimorbi*, or a bacillus not distinguishable from it by any of the tests we have applied, in the soil of regions where milksickness has never been known; we have found it in normal cow dung, on various grain and forage plants, and in some other locations. We have not been able to demonstrate that the bacillus is present in any greater abundance in the soil of milksick localities than elsewhere and altho it may appear remarkable to others as it does to us that a bacillus so widely distributed and with such highly distinctive characters as *B. lactimorbi* should have so long remained undescribed; nevertheless such seems to be the case.

If infection of grazing cattle with *B. lactimorbi* occurs, it therefore must depend more upon the occurrence of pathogenic races or varieties

than upon the presence of the widespread non-pathogenic or slightly pathogenic stock.

**Animal Experiments.**—Rabbits, guinea-pigs, dogs, cats, lambs, and calves have been used by us in experimental work.

**Rabbits.**—A young rabbit (R. 1), weighing about 400 gm., was inoculated intraperitoneally with 2 c.c. of heart blood from a heifer that had died of "alkali poisoning" five hours previously. The rabbit to all appearances remained entirely well for four days, at which time a certain listlessness was noted. On exercising the animal it quickly became exhausted and after running a short distance would fall over and then slowly struggle to its feet, panting vigorously. The muscular weakness increased during a period of about five hours after this condition was noticed, and the animal then died quietly. The autopsy disclosed a general exudative peritonitis, the exudation being whitish-yellow and fibrinous everywhere, all organs being more or less covered by it. The liver was very much enlarged, dark red, soft, and friable. On section the lobules could not be readily distinguished (cloudy swelling). The spleen was enlarged, deep red, and soft, the Malpighian bodies being barely discernible. The kidneys were enlarged, moderately red, and showed cloudy swelling. In portions of the small intestine the vessels were prominently injected, but there were no ecchymoses. The heart was apparently normal.

**Bacteria found:** Coverslip films made from the peritoneal exudate, heart blood, liver, and intestinal mucus showed in all cases long slender bacilli with the morphologic character of *B. lactimorbi*; in the intestinal mucus other forms were also present. Plates made from peritoneal exudate and heart blood gave pure cultures of *B. lactimorbi*.

A second rabbit (R. 2) was inoculated intraperitoneally with about 1.5 c.c. of the clot in the heart of R. 1 rubbed up in sterile water. Unfortunately this rabbit died during our return journey to Chicago (about 3 days after inoculation), and before we were able to make an autopsy decomposition changes had gone so far that the material was virtually worthless. Other rabbits (adults) have been fed with pure culture of *B. lactimorbi* and also inoculated subcutaneously and intraperitoneally, but up to the present with negative results.

**Guinea-pigs.**—A number of guinea-pigs have been fed and injected with pure cultures of *B. lactimorbi*, for the most part with negative results. In a few instances, however when animals were used that had previously been weakened by other treatment, for example, by injection with diphtheria toxin-antitoxin mixtures, death occurred. No characteristic changes could be certainly observed in the organs, but in one case (G.-P. 8.) *B. lactimorbi* was isolated in pure culture from the liver, plates made from the heart-blood and bile of the same animal showing no growth.

**Dog 1.**—From January 28 to March 5 this animal received agar and gelatin-plate cultures of *B. lactimorbi* from steer P. in nine doses. On March 8 it received three gelatin-plate cultures. This animal was a rather old dog, fat, lazy, and docile. Ten days after the first feeding it was noticed that its appetite was not so good as usual. Two days later it passed some masses of hard feces with a small quantity of bloody mucus. On examining this mucus bacilli were found in good numbers along with cellular débris. When the bacilli were stained with methylene blue they showed metachromatic granules, but there were no spores present. Plated in agar the material yielded the typical *B. lactimorbi*, and abundant spore-formation



was observed. Eight days later the animal again passed a considerable quantity of bloody mucus when out for exercise on the street in care of an attendant; no cultures were obtained, and it was not observed to pass any further mucus. Four days after the mucus was first observed the animal was noticeably thinner and showed well-marked irritability which as time passed developed into an almost maniacal raging when the cage was approached, altho when out for exercise it was quiet enough, except to actual strangers at whom it snapped when they attempted to pet it. February 27 it was in a decidedly weak and emaciated state. It also had a marked limp in the left forefoot, but on examination of the paw no foreign body could be found. On extending the leg evidence of pain at the shoulder joint was given, altho there were no external manifestations at that point. By February 29 the dog for the first time failed to leave its box voluntarily. On March 1 there was very definite weakness and stiffness in the hind legs. Its bark became harsh and husky and the conjunctivae were quite deeply injected. This condition marked the height of the clinical manifestations, and since the animal became no worse it was killed by chloroform on March 10 and an autopsy made immediately.

*Abdominal cavity.*—On opening the peritoneal cavity there are no signs of inflammation anywhere. The liver is definitely enlarged; general color a deep chocolate, in places somewhat mottled by lighter-colored areas; there is much blood in the organ, but on pressing this out the liver takes on a yellowish cast due to a moderate grade of probable fatty change in the parenchyma; consistency soft, capsule and liver substance rupturing easily on handling. On section the liver lobules are not clearly marked out and there is some cloudy swelling present as well as some fatty change; no focal necroses are seen; the tissue contains much blood; color red brown. The gall-bladder and bile seem to be normal. Spleen is probably normal in size; color dark red. Malpighian bodies near surface are readily seen; consistency is about normal. On section, it contains considerable blood, but one can detect the Malpighian bodies without difficulty. Kidneys appear to be normal, both outwardly and in section. Adrenal glands look normal and on section are pale. Small intestine is injected in some places, but not markedly so; Peyer's follicles look prominent as seen from the outside; the mesenteric glands are not enlarged or altered; large intestine is apparently normal and in places contains fecal matter. On opening the whole gut there is some injection of the mucosa of the small intestine, but in only a slight degree; no ulceration and altho Peyer's patches appear a trifle prominent there is no sign of inflammation in them. There is present a large number of a species of tapeworm. The large intestine shows no demonstrable lesions. No ecchymoses can be found in the serous membrane of the cavity. Bladder is not injected and is apparently normal.

*Thoracic cavity.*—No fluid or ecchymoses are present in the pleural cavities, and no signs of inflammation. Lungs are light in color and apparently normal. Heart is a trifle large for a dog of its size (20-lb. dog), walls of both ventricles are pale and soft; valves are free of inflammatory changes; endocardium is clear; pericardium is clear and shows no ecchymoses.

*Brain.*—Cortical vessels are somewhat injected and full; no signs of meningitis. On section, no injection of the vessels was found, ventricles appear normal, with a slight trace of clear fluid in them. Cerebellum and pons and medulla are seemingly in a normal state.

**BACTERIOLOGY.**—Coverslips and cultures in agar were made from the liver and kidney. The coverslips showed nothing characteristic; from the agar plates,



from both liver and kidney, *B. lactimorbi* was isolated in pure culture after 48 hours' growth.

**MICROSCOPIC.** *Liver*.—The whole section shows evidence of cloudy swelling, but more particularly are the cells of the middle and central zones of the liver lobules severely affected; the cells are greatly increased in size, take eosin strongly, and their protoplasm appears in minute round granules of colloid-like material and occasionally contains fat globules. Despite the protoplasmic changes the nuclei in general take the hematoxylin well; only occasionally do they appear vesicular and staining poorly, or are absent. Capillary vessels and central veins are full of blood and distended.

*Spleen*.—Slightly hyperemic; otherwise normal.

*Colon*.—Appears normal.

*Kidney*.—Very hyperemic; otherwise normal.

*Heart*.—Normal.

*Cerebrum*.—Normal.

*Small intestine*.—Hyperemic; otherwise normal.

**Dog. 2.**—A young dog of some fifteen pounds' weight, in good condition was received on February 16, and during the interval between this date and March 19 it received four doses of gelatin-plate cultures of several different strains of *B. lactimorbi*. On February 29 it was noticed that the animal had become noticeably thin, although lively and active. On March 10 it was much thinner and perceptibly weak, remaining contrary to habit in its box, after entrance of anyone into room. Next day it had developed a bronchitis, causing it to cough and interfering with its barking. On March 12 it was evidently very ill, lying in its box and showing great disinclination to rise; March 17 found it still in an enfeebled state, unable to bark, and the cough still continuing. On March 19, however, it appeared much better and was out of its box; the cough was less severe. On March 20, it barked vigorously; the cough had almost disappeared, and the animal was lively. It was killed by chloroform and an autopsy was performed at once.

Nothing of note is revealed by inspection. Reflection of the skin shows that the dog is exceedingly emaciated, there being no subcutaneous fat present. The muscles everywhere are very pale red. The inguinal and axillary glands are of normal appearance.

**Abdominal cavity.**—On opening the abdominal cavity the peritoneum shows no signs of inflammation nor ecchymoses. The liver does not appear to be enlarged; color light chocolate-brown; firm to the feel of the hand; it does not appear to show cloudy swelling or fatty change, and does not contain any excess of blood. No focal necroses are seen through the capsule. The gall-bladder is empty. On section the liver looks in a tolerably healthy state, as does the gall-bladder. The spleen is normal in appearance, both in the cut and uncut condition. In similar way the kidneys are likewise in an apparently good state. Adrenals are normal. The small intestines are found to be empty, and in places a bare trace of injection of the outer coats can be found. The large gut is seemingly in a healthy state and contains some fecal material. On opening up the intestinal tract, it is found that the small gut contains a fair amount of bile-stained mucus, and there is seen an occasional slight swelling of a Peyer's patch, but no sign of inflammation; there is an entire absence of any past or present sign of necrosis anywhere in the gut. The large gut contains soft feces and is in a normal state. Stomach apparently normal.

**Thoracic cavity.**—The heart looks of proper size and is not soft to the touch, but

the muscle of the ventricles is somewhat pale. Valves and endocardium are normal. The lungs show a few small points of atelectasis in the dorsal portions of the lobes of each; no trace of any bronchopneumonia. Trachea shows in its walls and in the wall of some of the larger bronchi some few flakes of muco-pus, altho there is lacking any of the signs of active inflammation. Brain and spinal cord are in a normal condition.

**BACTERIOLOGY.**—Coverslip preparations were made from the liver, intestinal mucus, and muco-pus of the trachea. That from the liver showed no bacteria; that from the intestine a number of colon-like bacilli and a few cocci; in the tracheal pus there were quite a number of lanceolate diplococci and a very few bacilli. Cultures in agar were made from the liver, intestinal mucus, kidney, and heart blood. Liver and heart blood sterile, the intestinal mucus gave *B. coli* and streptococci; *B. lactimorbi* was not obtained.

**MICROSCOPIC.** *Cerebrum*.—Appears normal.

*Cerebellum*.—Appears normal.

*Small intestine*.—Normal.

*Kidney*.—Normal.

*Heart* (cross-section).—Normal.

*Liver*.—Presents diffuse parenchymatous hepatitis of a moderately severe degree with considerable accompanying fat metamorphosis; the nuclear changes are not very great.

**Dog 3.**—This animal proved unsuitable for experimentation.

**Dog 4.**—This animal received gelatin-plate cultures of one strain of *B. lactimorbi* on March 19, 23, 29, and April 8. On April 7 it appeared to be less active in its movements, a trifle thinner, and showed no disposition to bark or whine. Next day, altho it barked a little, observers received the impression that it was somewhat enfeebled. On April 9 it was weak and refused to leave its box, and when persuaded to rise up in the box it trembled perceptibly in the forequarters and neck muscles. On April 11, it was lively and again got out of the box and danced about in its cage and wanted to be petted. On April 14 no signs of illness could be further detected. Chloroformed. Condition found on autopsy similar to that observed in Dog 2.

**MICROSCOPIC.** *Kidney*.—Glomerulitis; exfoliated cells, etc.; parenchymatous nephritis of a mild grade affecting the convoluted tubules and descending loop of Henle. Some of the descending tubules of Henle's loops contain considerable fat.

*Liver*.—The cells of the whole section are in a state of cloudy swelling particularly so in the middle and central zones of the lobules. The swelling of the cells renders the capillaries less conspicuous than normal. In two localities are seen foci of chiefly polynuclear leukocyte invasion, one much greater than the other, arising from the portal canals; where the leukocytes invade the lobules the liver cells have vanished.

**Dogs 5-8.**—(See article by A. B. Luckhardt, this *Journal*, p. 497.)

**Cats 1, 2, and 3.**—These kittens were fed, respectively, with milk from three cows that were on the Altamonte farm at the time of the outbreak (p. 430). The kittens were all puny and in poor condition. Nos. 1 and 3 received about 30 c.c. each, No. 2 about 20 c.c. No. 2 died about three days after the feeding began; the autopsy showed no pathological changes other than those consequent on inanition and anemia. *B. lactimorbi* was not found in the organs or intestinal contents. No. 1 died two days later and manifested a similar pathological condition. The bacteriological examination showed the heart, liver, and kidney to be sterile; the intestinal contents gave

*B. lactimorbi* together with common intestinal forms of microorganisms. No 3. died twelve days after beginning the feeding. The autopsy notes are as follows: "The only pathological condition (other than great emaciation and anemia) was in the large intestine. The whole intestinal tract was empty; the small gut contained a few round worms and much sticky mucus; there were no signs of inflammation; *the large gut from its beginning to within 3 cm. of the anus showed a patchy injection of the mucous coat which was somewhat edematous as well.* The liver was paler than normal, but was of normal consistency and showed no lesions of a macroscopic nature. The spleen seemed a trifle larger than normal and it was difficult to determine whether parenchymatous lesions were present. The heart showed no ecchymoses, but was paler than normal tho not of softer consistency. The lungs appeared normal."

**MICROSCOPIC.** *Liver.*—Throughout the whole section there are evidences of an existing mild degree of parenchymatous degeneration, the cells having contents of round granular bodies which give a general stain less pronounced than do normal cells; here and there are cells without nuclei, some poorly staining, whilst the majority stain well. In several parts of the section necrotic areas exist into and around which connective-tissue proliferation has taken place and round-celled infiltration; in one or two of these areas some necrotic liver cells can yet be seen. There is no hyperemia present and no cholangitis.

*Spleen.*—Apart from the presence of blood capillaries lessening the distinctive appearance of the Malpighian bodies the organ seems to be quite normal.

*Kidney.*—Practically normal; only occasionally is the evidence of a tubule or two showing evidences of parenchymatous degeneration.

*Heart* (cross and longt.).—In a few places in the specimen do the fibers retain cross striation; most of the fibers in certain bundles are shrunken and contain little stainable substance while their nuclei are swollen, distorted, and stain very poorly. In a number fat droplets can be discerned. In two places there are evidences of interstitial myocarditis with destruction of muscle elements in their locality.

*Colon.*—Much mucoid change and atrophy of cells of the tubules, with some round cell infiltration and superficial necrosis.

**BACTERIOLOGY.**—Three days after the milk diet had ceased and seven days before death, a swab culture was made from the rectum and resulted in the isolation of *B. lactimorbi*. At autopsy cultures were made from the small and large intestines, the liver, kidney, and heart blood, but the bacillus was not found.

**Cat 4.**—A young, active, perfectly healthy animal about 6 months old. Rectal temperature 99.6° F. August 12, fed two slant-agar cultures of *B. lactimorbi* in milk. August 13, apparently quite well. Rectal temperature 102.4. Feeding of August 12 repeated. August 14, appears lively and well. Temperature 101.3. Dosed as before. August 15, very lively but thinner. Temperature 103.4, received same dose as before. August 16, temperature 103.6, mucus noticed on thermometer after withdrawal from rectum, anus reddened. From this date to August 30 (animal killed) daily doses of the same culture were given except on these dates, viz.: August 24, 27, and 28. August 17, temperature 102.6. August 18, slight redness of rectum, no mucus; swab cultures made from rectum evidently caused pain. August 19, lively and active; temperature 102.2; anus considerably reddened; signs of pain when thermometer inserted. August 20, animal shows sign of irritability, is not so active, shows notable weakness of hind legs; temperature 102.4. August 21, temperature 102.6.

August 22, kitten lively and able to run about altho experiences marked difficulty in springing up due to weakness in hind legs; temperature 101.6. August 23, kitten very lively, redness about anus gone, muscular weakness barely perceptible; temperature 101. August 29, temperature 102. August 25, kitten appeared normal, temperature 99. August 26, kitten thin but frolicsome; from the anus there exuded for the first time bloody mucus. Coverslip preparations showed multitudes of morphologically typical polar-staining bacilli thought to be *B. lactimorbi*. Culture showed definitely the presence of this organism. August 27, temperature 98.4. August 28, temperature 99.6. August 29, temperature 99.6. August 30, since August 26, the kitten has been well and lively. Chloroformed and autopsy made; the body seems perfectly normal. Cultures from heart blood and liver were negative (sterile). Kidney and liver preserved in 10 per cent formaldehyde.

**MICROSCOPIC.** *Kidney*.—Occasionally are found convoluted tubules that show parenchymatous degeneration accompanied by fatty accumulation in the shape of small droplets. In the descending limbs of Henle's loops much the same picture is seen only that the fat drops are relatively large, and at times are often present where no parenchymatous degeneration exists. Glomeruli normal.

*Liver*.—Generalized parenchymatous hepatitis, in places within the mid or central zones taking on an intenser focal reaction. Accompanying this condition is marked fatty metamorphosis, the fat being usually distributed in the cells as small droplets; in one locality some of the lobules looked like adipose tissue, so much fat did the cells contain. No necrosis found, only a couple of points of round and polynuclear leukocytic infiltration. No cholangitis. No hyperemia.

**Cat 5.**—Owing to condition of this animal feeding experiments were not undertaken.

**Cat 6.**—January 16, weight 990 gm. Fed 10 gm. suspected butter (from Carlsbad, N. M.) in warm milk. *B. lactimorbi* was isolated from this sample. No change noted, dose repeated. January 18, dose repeated. No signs of illness. January 19, looks thin and out of condition; weight 990 gm. January 20, has bronchitis; playful, but weak and emaciated; weight 930 gm. January 21, animal found dead in morning, body placed on ice by janitor, but notification delayed. Autopsy at 10:30 A. M., January 23. Body in an excellent state of preservation. The animal showed great emaciation, there being no subcutaneous fat. On opening the abdomen no signs of peritonitis. Liver appeared somewhat enlarged, was not as dark colored as usual, and in many places the lobules were not discernible. It was not particularly friable. On section, the organ did not contain much blood, the lobules were indistinct or not discernible, there being apparently some moderate degree of cloudy swelling. The spleen was dark colored, a trifle soft, and enlarged; on section contained blood; Malpighian bodies not seen. The kidneys seemed somewhat enlarged and waxy looking. On section, the cortex of each was yellowish-white in color, apparently fatty, blood vessels between the bundles plainly seen; the Malpighian tufts not seen. The small intestines, beyond containing much mucus, appeared normal. Large intestine normal. Mesenteric gland at ileocecal-colic region enlarged and pale. Adrenals seemed normal.

**Thorax.**—Heart: Right side contained much blood; left empty. Muscle looked pale and was soft. Valves normal. No ecchymoses. No pericarditis. Lungs: Both alike, voluminous; only the upper part of the upper lobe appeared normal. The rest was of a dull, light, muddy, red color throughout and showing small roundish gray spots. On feeling the tissue there was no crepitation noted, a feeling of solidity was

everywhere manifest, pieces cut from these lobes sank at once in water. The cut surfaces were smooth and pus oozed out of cut bronchi in places. There were no signs of pleuritis and no ecchymoses.

**MICROSCOPIC.** *Liver*.—The organ is hyperemic and the blood everywhere contains a large number of round and polynuclear cells. The marked feature in the specimen is the general normal appearance of the cells but here and there throughout the section small, marked focal necroses, in many places no parenchymatous cells remaining, accompanied by infiltration of leukocytes of the round and polynuclear variety. The lymphatics of the portal canals are crammed with leukocytes. As a rule there is no destruction of bile ducts by the necrotic process; in one or two places such has occurred by the spread of the inflammatory reaction from a nearly necrotic focus in the liver substance.

*Kidney*.—Normal.

*Lung*.—Very edematous, alveolar cells desquamated in excessive numbers accompanied in a few places with a moderate number of white blood cells; as a rule these latter are everywhere scant in numbers. Hyperemia. No bronchitis, altho in bronchioles filled with fluid some of their epithelium has desquamated.

*Spleen*.—Very hyperemic. Malpighian bodies stand out sharp and normal.

**Cat 7.**—This animal was fed like Cat 6 with butter from New Mexico. The butter in this case was not definitely implicated in the production of illness, altho coming as it did from a region where milksickness prevailed it was regarded as possibly infectious. *B. lactimorbi* was found in the butter. The cat remained well.

**Cat 8.**—Fed with butter presumably concerned in the production of the outbreak of milksickness at Morris, Ill., November, 1908 (p. 416). Conditions of experiment as with Cat 6. *B. lactimorbi* found by culture to be present in the butter. Three doses of butter of 10 gm. each given in warm milk on January 16, 17, and 18. Weight January 18, 990 gm.; January 19, 985 gm.; January 20, 965 gm.; January 22, 880 gm.; January 23, 865 gm.; January 24, 820 gm.; January 25, 8—gm.; January 26, 765 gm.; January 27, dead. During this period there was little noticeable change until January 24, when the cat, altho eating well, was noticed to be getting very weak. This was followed by rapid emaciation and death.

*Autopsy*.—A much emaciated animal. No subcutaneous fat. *Lung* partially collapsed, both alike in appearance. Lower lobes congested and showing a few scattered areas of bronchopneumonia; portions cut from congested areas float. No ecchymoses; no pleuritis. *Heart* apparently normal as were *all other* organs; the mucosa of the small intestine showed faint general injection; gut empty.

**MICROSCOPIC.** *Small intestine*.—Normal.

*Kidney*.—Cells almost everywhere have a peculiar hyalin appearance (faulty fixation).

*Spleen*.—Hyperemia and an increase in cells of spleen pulp. Malpighian bodies normal.

*Lung*.—The section in part shows collapse of the alveoli, accompanied by leukocytic invasion in two localities with bronchial involvement (bronchopneumonia). General hyperemia.

*Liver*.—General hyperemia accompanied by a leukocytosis. Parenchymal cells generally normal; here and there some lobules show fatty change and cloudy swelling; and occasionally are found very small foci of necrosis with accompanying leukocytic infiltration.



**Cat 9.**—Fed with a filtrate of a four-months-old asparagin-medium culture of *B. lactimorbi*; negative result.

**Cat 10.**—Fed with asparagin solution alone as a control; negative like Cat 9.

**Lambs.**—These animals were subjects of a feeding experiment extending over about three weeks. They were given gelatin cultures of two strains of *B. lactimorbi*, one from feces of A. McC., Altamonte, Ill., the other from the brain of J. Morrow's lamb (No. 1), Tiro, O. Through a mistake on the part of the attendant, both lambs were given mixtures of the cultures, so that independent pathogenicity of two strains was not established. No definite signs of illness were noted.

**Lamb 1.**—Male, about six months old, weight about 35 lbs. Chloroformed, and autopsied at once. There was little subcutaneous fat on reflecting the skin.

**Thoracic cavity.**—No abnormal amounts of fluid in either pleural sacs or pericardial sac. The thoracic viscera appeared quite normal.

**Abdominal cavity.**—There was no general peritonitis. The rumen evidently contained food as it was distended some; the other three portions of stomach seemed empty. The upper two-thirds of small intestine was empty and pale; venules distended with blood but showing no inflammatory condition. The lower one-third of gut, the cecum, and the colon contained fecal material.

In four places on the peritoneal surface of the lower one-third of the small gut were found yellow masses of fibrino-purulent nature about 4 mm. in diameter, with no signs of inflammation about them. These were smooth on top, and could be peeled off easily *en masse* from the tissue below without causing loss of substance. On account of its tough nature, and lack of inflammatory reaction about it, the exudate was considered old. Each cap of exudate was found to have underneath it, localized in the wall of the gut, an opaque, yellowish-gray, tough nodule. Other nodules could be seen and felt in other places in the gut and in the cecum without their being capped with old exudate. On opening the intestine, it appeared normal with the exception of the aforesaid nodules which were found to be imbedded in the submucosa, being covered with intact mucous membrane, and showing no evidence of present inflammation; the process seemed to be undergoing resolution. The colon was free of nodules and looked normal. The mesenteric glands were pale and enlarged and firm; one particularly large gland was found close to the portal vessels.

**Liver.**—The organ did not seem to be enlarged. On its upper surface but in the parenchyma were found two areas of a yellowish-gray color, about 1.25 cm. in diameter, projecting above the surface about 3 mm. in a nodular manner, capped with exudate similar to that seen on the surface of the small intestine, but in one spot being lightly glued to the diaphragm over an area roughly 4 cm. all around the nodule. The exudate was old and there were no signs of acute inflammation in or around the nodules. A similar nodule was found on the under surface of the liver near the free margin and 3 cm. to the left of the gall-bladder. The liver was of a deep red color before being incised and cut free; on bleeding it was found to be of moderately deep ochre color; the lobules could be made out, there was no evidence of fatty change, nor could there be said to be any very definite evidence of cloudy swelling, altho the consistency of the organ was less than normal. Gall-bladder normal. Spleen normal. Kidneys normal. Heart normal. Brain and meninges normal.

**BACTERIOLOGY.** *Agar-plate cultures* made from heart, liver (nodules and exudate above them), intestinal (exudate over nodules; portal gland). Coverslips from same locations excepting heart blood.

**MICROSCOPIC.** *Liver.*—(a) The whole section shows condition of cloudy swelling of mild degree associated with fatty metamorphosis; the fat granules being numerous within each cell, occasionally they are of large size and few in each cell. The degeneration is less marked in the outer zone of each lobule. The nuclei of the cells stain poorly or are absent. The enlargement of the cells almost obliterates the capillaries and there can be found here and there in-growth of connective-tissue cells in few numbers from the portal canals into the lobules; in other places it seems to arise from the capillary endothelium. In no instance is this proliferation more than barely noticeable. Central veins are well filled with blood altho little can be seen in the greatly compressed capillaries. In places the capsule shows signs of inflammatory reaction with granulation-tissue formation. The liver cells for a short distance below the capsular reaction are extraordinarily increased in size and show cloudy swelling in marked degree.

b) This section was taken from around one of the necrotic foci; it shows a large irregular necrotic mass of tissue, which has a feeble architectural resemblance to liver substance in parts (contains broken nuclei, etc.) surrounded on all sides by active inflammatory tissue, made up of large numbers of round cells, fewer polymorphs, and a large amount of granulation tissue, some few multinuclear cells (not like tubercle) and new capillary vessels. The amount of liver substance in the section shows cloudy swelling with fatty changes, and there is marked round-cell infiltration and connective-tissue proliferation in the portal canals and apart from them in places. No bacterial masses (high-power dry lens) can be seen associated with the process, not even in one very small focus; there simply seems to be an acute focal necrosis, intense focal karyorhexis, and, outside of this, granulation-tissue formation more or less free of cellular infiltration but bounded by a dense zone of round cells.

*Small intestine* (section through nodule).—There is a generalized intense inflammatory reaction involving the mucosa of the whole circumference of the section, the villi being greatly thickened and swollen by the congested blood vessels and masses of round cells and occasional polynuclear leukocytes. There is great destruction of Lieberkühn's follicles in many places, in others infiltration with cells. The lymphatics piercing the inner muscular coat contain round and polynuclear leukocytes. In particular the inflammatory reaction is concentrated in three foci of acute inflammation accompanied by relatively extensive necrosis, two foci are close together, and the third a little distance away. They have arisen in the submucosa and each is surrounded by an actively proliferating zone of granulation tissue containing some round and polynuclear leukocytes, and beyond, greater numbers of these cells are located. The necrotic tissue seems to have been made up of round cells and proliferated connective-tissue cells. The inflammation in one place extends through the submucosa toward the lumen of the gut and is covered by a thin layer of mucous tissue devoid of any tubular structure; only a few shrunken villi remain and these are denuded of epithelium and are hyperemic. In another focus the inflammatory reaction has pushed through the muscular coats and has involved the peritoneum in acute inflammation. No bacterial masses can be made out by high-power dry lens.

In other sections taken where no nodules were found the gut is normal.

*Heart* (long. sect. vent.).—Everywhere the muscle retains its striations altho the fibers in many bundles do not take the stain well. There is a very general acute interstitial myocarditis as shown by the displacement of fibers by accumulation of large numbers of both round and polymorphonuclear leukocytes and proliferation of endothelial cells.

**Kidney.**—Acute parenchymatous nephritis affecting the convoluted tubes chiefly, in less degree the loops of Henle, whilst only occasionally the collecting tubules; fatty deposition seen in scant degree here and there. Colloid material seen now and then in some tubules. Necrosis of epithelium. The glomerules simply show evidence of hyperemia. There are no interstitial changes.

**Lymph gland.**—Hyperplastic and edematous.

**Lamb 2**, male, about six months old, weight about 28 lbs. Chloroformed at 3:20 P. M., and body examined at once. Conditions were practically same as in Lamb 1 excepting that there were fewer capped intestinal nodules, but more imbedded ones in the same regions and particularly in the cecum. The liver had only one surface nodule, but several internal foci or nodules which as in the former case showed no signs of active inflammation and were also tough to the knife. The consistency was much softer, the lobules could not be made out, and there was evidence of cloudy swelling, but no fatty change. Color a dark ochre.

The general impression received was that of an infection of the intestines and liver that had some time since passed the acute stage and had already advanced along one of general resolution.

Other organs as named in Lamb 1 were also found to be normal.

**BACTERIOLOGY.**—Cultures (agar plates) were made from heart blood, liver nodules, mesenteric gland, cecal submucous nodule, cecal feces, and purulent cap over cecal nodule.

*Culture results, September 2:*

**Lamb 1.** *B. lactimorbi* present in heart blood, and liver nodule (*abscess*); *Staph. albus*, portal gland; *Sterile*, exudates on liver and intestinal nodules.

**Lamb 2.** *B. lactimorbi* present in heart blood, cecal feces, exudate over cecal nodule, mesenteric gland, and in cecal nodule; *Bacillus* (undetermined),<sup>1</sup> liver nodule.

**MICROSCOPIC.** **Kidney.**—As for Lamb 1.

**Liver.**—Quite similar to section (a) Lamb 1. In addition there is a local perihepatitis nodular in character. The node is made up of granulation tissue, center necrotic, and necrosed portion apparently in the midst of a sero-purulent fluid. The granulation tissue contains round and polynuclear leukocytes and is surrounded with a zone of the same. The tissue on the extreme periphery of the focus of acute inflammation is edematous. A slight amount of the liver tissue has been involved in the inflammation, and certain of the liver cells near by seem to be undergoing proliferation, showing intense staining of nucleus, mitosis, dense staining of protoplasm.

**Large intestine.**—Great hyperplasia of solitary follicles accompanied by a very considerable degree of both round and polynuclear leukocytic infiltration of submucosa and mucosa, destruction and loss of columnar epithelium of tubules and tubules themselves in the inflammatory areas. Another section passes through a nodule in the walls of some portion of the large gut. The nodule appears to be a greatly hyperplastic solitary lymph follicle.

**Pancreas.**—Nothing abnormal detected.

**Calf 1.**—A calf (C. 1) weighing about 350 pounds, was fed with agar-plate cultures of *B. lactimorbi* at intervals of a few days during a period of about six weeks. To all appearances the animal remained entirely well and showed no characteristic symptoms, nor, indeed, symptoms of any kind. The animal was killed at 9 P. M., and the autopsy

<sup>1</sup> Culture accidentally lost.

was held the next morning at half-past nine. The following pathological conditions were found:

*Heart*.—A small ecchymotic spot was found beneath the epicardium of the right ventricle near the apex; the organ was otherwise in an apparently normal condition.

*Liver*.—On the surface in three or four places were found small areas of irregular shape of a uniform reddish-gray color in which the architecture of the liver substance could not be seen. These were considered to be necrotic in nature. On section, these areas seen on the surface were found to dip down into the parenchyma to varying depths, preserving the same features as seen on the surface. On further examination of the organ there was found in the posterior portion of the large lobe some 3 cm. below the surface an irregularly shaped necrotic mass of about 6 cm. in diameter, whose appearance corresponded entirely to that of the smaller areas just described: it was very friable, breaking down on the slightest pressure. In other respects the organ appeared normal.

*Mesenteric glands*.—These were generally much enlarged and less firm than normal. Other organs and tissues appeared to be in a normal state.

**MICROSCOPIC.** *Lung*.—Bronchial lymphatics crowded with round cells, no sign of tubercles. Lung otherwise normal.

*Lymphatic gland*.—Normal.

*Spleen*.—Apparently an increase in the fibrous-tissue elements of the organ.

*Kidney*.—Acute parenchymatous nephritis confined most largely to the convoluted tubules, and the ascending and descending loops of Henle, much less in the collecting tubules. The tubules contain both granular material and colloid droplets. There is glomerulitis also, with some desquamation of cells and collection of granular and colloid material within Bowman's capsule.

*Heart*.—Specimen shows normal tissue.

*Liver*.—Postmortem change only; invasion with *B. welchii*.

*Colon*.—Appears normal.

**Calf 2**.—Another animal (C. 2) was fed with spore-bearing cultures in a similar manner. Three days after the first feeding the feces were soft, unformed, and contained much mucus. When exercised by running the animal was readily fatigued and showed great unwillingness to move, altho on the preceding day it had been very active. On the fourth day the disinclination to muscular exertion was still more marked. There was much mucus in the feces, and microscopic examination showed the mucus to be filled with slender, granular-staining bacilli morphologically agreeing with *B. lactimorbi*. With the use of cultural methods *B. lactimorbi* was isolated and definitely identified. Nine days after the first feeding an abundance of blood-streaked mucus appeared in the stools. During the next two weeks little change was noted in the condition of the animal, beyond a slow but steady drop in the temperature, which after the first 10 days, ranged below 100° F. a large part of the time. Four weeks after the dosing was begun the animal was alive, but emaciated and rather stupid. At the end of another week while being led about the yard, the animal's forelegs seemed to give way suddenly and it went down on its knees with head on the ground, but did not lose its balance completely. After a while it struggled to its feet and moved on, only to repeat the performance a little later. This behavior strikingly reminded us of the cattle affected with "slows" or "tires," as we have seen them in the Pecos Valley, but there were no "trembles" in this case. The calf recovered and was chloroformed.

The autopsy was made at once. The only pathological condition found was

confined to the small intestine which was covered with small hard nodules averaging in size that of a pea (some were about the size of shot); these were found along the whole length of the intestine but were more plentiful in the jejunal portion. Some of the nodules were injected more or less deeply. The interior of some consists of a dry cheesy substance. The contents of the intestine were made up of much yellowish mucus and a little food material.

**BACTERIOLOGY.**—*B. lactimorbi* was isolated from the intestinal mucus and from the contents of a nodule, in the latter instance accompanied by a yellow coccus. The liver culture was sterile.

**MICROSCOPIC.** Alcohol fixation; hematoxylin and eosin. *Small intestine.*—Mucous membrane everywhere normal. Peritoneal covering thickened by increased growth of connective tissue, non-inflammatory. The section goes through a nodule which is made up of vascularized connective tissue showing no signs of any inflammatory change or degeneration; the peritoneum is everywhere intact.

*Cerebrum.*—Appears normal.

*Kidney.*—Poorly preserved.

*Spleen.*—Appears normal.

*Cerebellum.*—Appears normal.

*Heart muscle.*—Appears quite normal.

*Liver.*—Appears quite normal.

*Lymphatic gland.*—Quite edematous in its central portion, otherwise normal.

#### SUMMARY AND CONCLUSIONS.

There is no doubt that a disease exists under the name of milksickness, trembles, etc., which is common to man and some of the higher animals and is characterized by a fairly definite symptom-complex. This affection occurs occasionally at the present time in the states of North and South Carolina, Kentucky, Tennessee, Ohio, Illinois, Indiana, Michigan, and Texas, and in the territory of New Mexico. It has prevailed in the central western states for upward of a hundred years; from 1800 to 1840 it was quite common in certain districts altho the actual area affected does not seem to have been much if any greater than at present. Its reduction seems to have been brought about by the discovery that certain tracts of land were concerned in the production of the disease and that clearing, drainage, or cultivation of these tracts removed their dangerous qualities. "Milksick land" has also been fenced off from adjoining pastures known to be harmless.

The disease is usually contracted in the first instance by grazing cattle or sheep having access to the infected territory. It is communicated to man most frequently through the medium of raw milk or butter; meat also, especially if not thoroughly cooked, is thought



to convey the disease. Carnivorous animals are liable to develop the malady when they feed on the carcasses of animals dead from trembles. Water is a possible source of the disease in both man and animals.

The course of the disease in cattle is marked by lassitude and muscular weakness sometimes, but not invariably, accompanied by constipation. There is often muscular twitching or trembling and occasionally signs of nervous excitement. The temperature in the mid-course and late stages of the disease is generally normal or subnormal.

In man there is as a rule excessive vomiting and obstinate constipation accompanied by great weakness. The temperature is normal or subnormal. A peculiar sweetish odor, which is also noticed in cattle and is due to acetone, is present in the breath and in the urine. The recorded mortality in man is about 20 to 25 per cent but owing to the fact that the severe cases have attracted most attention the actual mortality is probably much lower than this and is very likely less than 10 per cent. Relapses are rather frequent. Little if any immunity is conferred by an attack.

One of the main lesions found in the postmortem examination of cattle is fatty degeneration of the liver which is often very extreme. The small intestines are usually empty except for a tenacious yellow mucus. In some instances hyperplastic areas are noticed in the intestinal walls and occasionally small hard nodules are found. The mesenteric glands are often enlarged. The kidneys show as a rule cloudy swelling or fatty degeneration. We have found no involvement of the central nervous system. The heart muscle often shows fatty degeneration, sometimes in slight, sometimes in marked, degree. Ecchymoses on the wall of the heart, on the liver, and occasionally on the spleen are observed. The voluntary muscles do not seem affected.

In man the changes observed by us in the autopsy of a single fatal case are very similar to those found in cattle. Tissues from other fatal cases show microscopically extreme fatty degeneration of the liver, and a similar alteration or cloudy swelling in the kidney. We have found nothing resembling the profound disturbance of the central nervous system described by Graff.

Many different theories have been advanced to account for the origin and peculiar characters of the disease. Mineral poisons, such as arsenic and copper salts, have been conjectured to exist in certain soils in such quantities as to be taken up by forage plants which in turn are eaten by grazing animals. The poisonous substances are then supposed to be eliminated in the milk and to cause poisoning in man when the milk or milk products are taken into the alimentary canal. Analyses, however, have failed to reveal the presence of foreign mineral substances either in the suspected food substances, in the stomach contents or the tissues of affected animals, or in the soils of the incriminated regions. There is furthermore no correspondence between the underlying geological formations or the chemical composition of soils and the presence or absence of the disease. Adjoining tracts of pasture land to all appearances identical in character may on one side of a division fence give rise to milksickness, on the other be harmless.

Poisonous plants of various kinds have been held by observers to cause milksickness. There is no agreement, however, as to the responsible species. Plants firmly believed in some localities to cause the disease do not grow in other localities where the disease is quite prevalent. Feeding experiments with suspected plants have in no case given unambiguous results.

Living microorganisms or their products have been held to be the cause of the disease. According to the testimony of a number of observers the disease is self-propagating under some conditions and may be passed on without limit from one animal to another by the simple expedient of feeding one animal on the carcase of its dead predecessor. We have not ourselves been able to observe such a transfer, but our material has been very limited and observations of the disease under natural conditions show that transmission occurs very irregularly. It is also asserted that the virus of the disease is destroyed or weakened by heat in the same manner as that of various well-known infectious diseases. Observers agree that a single dose, and that not a large one, of affected milk or butter will produce the disease. Epidemiologically the correspondence of this disease with anthrax is very close.

From practically all cases of the disease that we have seen in man

and other animals (horse, sheep, cattle) we have isolated an aerobic spore-bearing bacillus which grows well and characteristically on the ordinary culture media. We have found this bacillus—named *B. lactimorbi*—in the internal organs and heart blood of animals examined but a few hours after death. In some instances it was present in pure culture (e. g., heart blood of a heifer, liver of a fetal calf, gut nodule of a horse, etc.). The same bacillus has been found by us in cow's milk collected by one of us under aseptic conditions and in butter suspected of having communicated the disease. It was also present in the feces in several non-fatal human cases of milksickness. We have also found it in the soil of milksick regions. The force of such observations is weakened by the fact that a bacillus to all appearances morphologically and culturally identical has been isolated by us, not only from the soil of districts where milksickness prevails and on certain plants growing in those regions, but also from earth taken from places where the disease has never been known, and from normal cow feces.

When this bacillus is injected subcutaneously or intraperitoneally in pure culture into rabbits or guinea-pigs little or no effect is usually produced, altho in a few instances it has seemed to exert some pathogenic action. Fed in large quantities to these animals no deleterious action whatever is observed. On the other hand when fed in considerable quantities to dogs and cats symptoms and lesions are produced that are very similar to those observed in animals naturally affected with milksickness. In dogs, for example, a tendency to bronchitis is manifested (cf. McCall, 1822). The lesions in experimental animals in some respects resemble macroscopically and microscopically those observed in animals that have contracted the disease in a natural manner. Lambs fed with this bacillus showed nodules in the intestinal wall similar to those found in some naturally infected animals. From the majority of experimental animals *B. lactimorbi* was recovered, often in pure culture, from the various internal organs such as the liver, spleen, and kidneys. It was also found in abundance in blood-flecked masses of mucus voided by the dogs and calves under experimentation. Two calves and a horse fed with cultures have given generally inconclusive results.

Taken as a whole the facts do not surely indicate that a specific

microorganism is the cause of milksickness or trembles. The bacillus in question belongs to a group which seems widely distributed and for the most part certainly not endowed with pathogenic qualities. In particular localities, however, where the soil conditions are favorable in respect to moisture and other unknown factors, strains of the bacillus perhaps possess pathogenic or toxigenic characters but if so, these, according to our experience, are quickly lost under artificial culture. It seems possible, judging from the observations of Mr. Luckhardt, which follow this paper, that *B. lactimorbi* is more commonly present on the surfaces of certain plants than on others but further observations will be needed to establish this fact.

The partly incomplete results and conclusions set forth in this paper are presented at this time with some hesitation, but the great rarity of material available for studying milksickness makes it desirable to record such facts as we have secured in the hope that they may be of use to other observers who chance across the malady from time to time.

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## ADDITIONAL NOTES ON THE BACTERIOLOGY AND PATHOLOGY OF MILKSICKNESS.\*

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INASMUCH as the literature on milksickness has been reviewed by Dr. Jordan and Dr. Harris in the preceding paper, such an introduction to the present paper is not necessary. Early in the autumn of 1908 I began at the suggestion of Dr. Jordan<sup>1</sup> the experimental feeding of dogs with what was believed to be the specific germ of milksickness. Since milksickness is primarily a disease of horses and cattle and is undoubtedly of intestinal origin I almost simultaneously instituted a search for *B. lactimorbi* on the various forage plants which serve as chief nutriment for these animals. This search was in a great measure successful. Various strains of this bacillus isolated from grasses and weeds were compared with the strains isolated by Jordan and Harris from the organs and tissues of animals dead and sick from milksickness, to determine whether possibly certain cultural differences might be revealed which would more or less sharply differentiate "pathogenic" from the widespread "saprophytic" varieties or strains. During this study certain cultural and biochemical features not described in the previous papers were observed. These new features together with the rather limited study of the distribution of the organism in nature and the technic adopted in isolation are embodied in the first half of the present paper. The latter half of the paper presents the results of the experimental work on dogs.

### BACTERIOLOGY.

**Distribution of the Organism and Manner of Isolation.**—The organism was isolated with the greatest ease from two samples of dried alfalfa from Wisconsin ("A. H." and "A. K."); from dried alfalfa

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<sup>1</sup> I wish to thank Dr. E. O. Jordan for helpful suggestions and encouragement during the progress of the work.

from Illinois ("A. M."); from a sample of very old dried alfalfa from Indiana ("A. I."); from *Bigelovia*, *Solanum elaeagnifolium*, *Gutierrezia sphaerocephala*, and *Portulaca pilosa* from the Pecos Valley, New Mexico; and twice from samples of Roquefort cheese. Timothy hay, red clover, bran, hominy, and silage gathered from the same farm from which the alfalfa showed practically nothing except *B. lactimorbi*, were examined bacteriologically for *B. lactimorbi* with negative result. A sample of alfalfa from Oregon where milksickness has never been known to exist showed no organism in any way similar to *B. lactimorbi*.

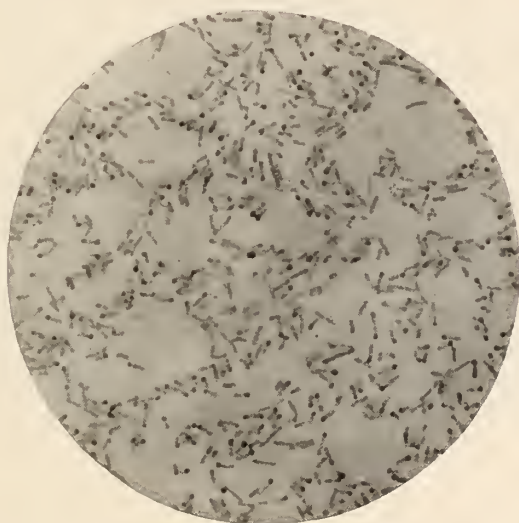


FIG. 1.—*B. lactimorbi* from Indiana alfalfa; 18-hour growth on agar at 30° C. Löffler's methylene blue.  $\times 1100$ .

The following method was adopted in isolating the organism from plants, e. g., from alfalfa. The more finely divided particles of the dried hay were carefully strewn over the surface of a slant agar tube taken directly from the sterilized bottle in which the samples were collected. The tube inoculated in this manner was then incubated at 30° C. for 18 to 24 hours. At this temperature *B. lactimorbi* if present outgrew the other organisms from the alfalfa so that the dull semi-transparent film-like growth which spread out from the edges of the small particles of the hay consisted almost entirely of

a pure culture of *B. lactimorbi*. When a coverslip preparation from this film-like outgrowth was stained with methylene blue, the identity of the organism was easily determined by the presence of the characteristic oval drumstick spores and the presence of metachromatic granules (Fig. 2). The metachromatic granules are particularly large and distinct at the time of and for a short period after isolation (Fig. 3). A loop of this growth was transferred to broth and immediately plated out in plain agar. The growth on the plates showed the organism to be present in almost pure cul-

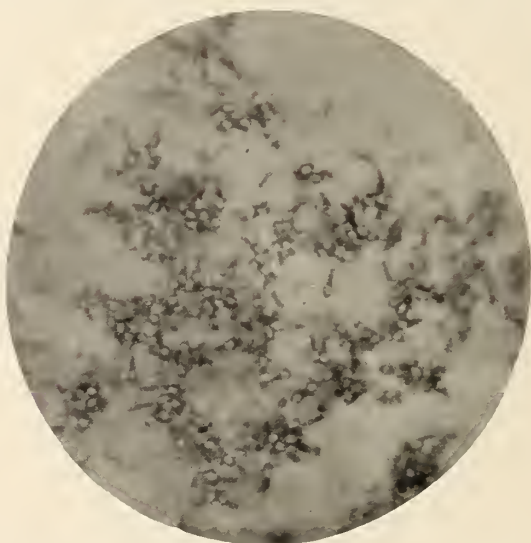


FIG. 2.—*B. lactimorbi* from *Bigelovia rusbyi*, Pecos Valley, N. M.; 24-hour growth on plain agar at 30° C. Löffler's methylene blue, no heat.  $\times 1150$ .

ture. After isolation in pure culture each strain was transferred to the ordinary culture media and the reactions observed compared with the cultural features and biochemical reactions of the organism as described by Jordan and Harris in their first paper.<sup>1</sup> On the whole the reactions were the same. Such differences as were observed together with additional reactions not noted in the previous descriptions will be mentioned here.

*Agar slant.*—Not all strains when grown at 37° or 30° C. show a “delicate veil-like growth” which more or less irregularly covers the surface of the agar. The growth may be entirely confined to the needle track as in the case of the strains isolated from

<sup>1</sup> *Jour. Amer. Med. Assoc.*, 1908, 50, p. 1665.

*Bigelovia* and *Gutierrezia sphaerocephala*; or if spreading, the growth may be thick and almost viscid and as regards luster can be easily mistaken for *B. subtilis* (Strain: "Por." and "Sol.").

*Litmus milk*.—Altho coagulation of the milk is not observed with most strains, the strain isolated from "Portulaca," "Solanum" and the strain "A. I." coagulated milk within 72 hours. The reaction of the milk was strongly alkaline. The strain isolated from *Bigelovia* produced in milk a small amount of a gelatinous sediment. With the increasing growth of the organism and the consequent increase of free alkali production the casein again goes into solution, giving the milk a brownish semi-translucent appearance. If flasks containing 50 c.c. of plain milk are inoculated and incubated for three weeks or longer at 30° C. the milk turns a light pink or dirty brown color. Such cultures give off a putrescent, rancid, and sour odor which in some instances closely

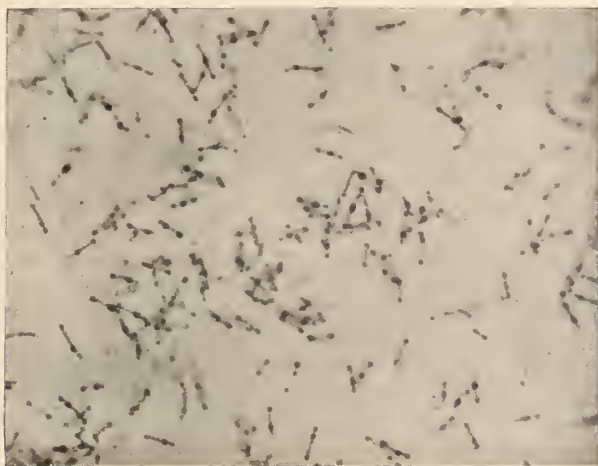


FIG. 3.—*B. lactimorbi* from Meeker alfalfa; 1st generation, 18-hour growth on agar at 30° C. Löffler's methylene blue, no heat.  $\times 1100$ .

resembles the odor of butyric acid. The milk cultures were repeatedly tested for acetone by means of Gunning's iodoform test, for it was thought possible that the rapid emaciation with the occurrence of acetone in the breath and urine during milk-sickness might be due to the decomposition of the fats of the body into acetone or acetone-like bodies through the agency of *B. lactimorbi*. The tests were, however, negative. The great alkalinity produced in the milk is due to the decomposition of the proteins of the milk with the production of free ammonia.

*Potato*.—In their first communication Jordan and Harris reported the absence of growth of the organism on potato. At that time undoubtedly the organism was of true parasitic nature as regards its food supply and found ordinary potato an unsuitable medium for development. Since then I have had no trouble in obtaining a good and in most instances a luxuriant growth on potato with the original strains and with all strains since then isolated by Jordan and Harris, and myself.<sup>1</sup> In general there appears

<sup>1</sup> This may be due to age or variety of potatoes used.

after 24 hours' incubation at 30° C. on the surface of the potato a greyish-yellow to reddish-brown (depending on the strain) moist growth which spreads slightly from the needle track. Even at this early stage there is noticeable a slate-blue or brownish discoloration of the medium which increases until on the sixth day the entire medium is discolored. Growth is usually accompanied by the production of a very rancid sour odor, tho this is not a significant feature. Smears made from the potato cultures on the sixth day show numerous involution forms with large metachromatic granules similar to the involution forms found after six days' growth of the organism on Löffler's blood serum. Large metachromatic granules, extruded from the cell body, were found massed together in large clusters. At other times minute metachromatic granules were irregularly scattered throughout the greatly elongated and coiled bacterial cell. The granules take an intense blue or reddish stain when treated with methylene blue or with Neisser's stain.

*Heinemann's synthetic potato medium.*—A good visible growth could be made out at the end of six days. The amount of growth naturally depended on the strain under investigation, being directly comparable to the luxuriance of the growth of the same strain on more favorable culture media. The growth was moist and glossy and had a yellowish to a brownish coloration. The growth was in the majority of the cases strictly confined to the needle track. There was no discoloration of the medium.

*Fermentation tubes with sugar broths.*—The organism grows equally well in plain, dextrose, lactose, and saccharose broths. At the end of 24 hours' incubation at 30° C. the medium in the bulb is uniformly turbid. Scum formation is pronounced. No growth takes place in the closed arm of the fermentation tube.

*Nitrate broth.*—Eight strains were grown in nitrate broth to determine whether the organism possessed the power of reducing nitrates to nitrites. The result was negative.

*Indol.*—Growth of the organism in standard peptone solution failed to reveal the slightest trace of indol production at the end of the tenth day and at the end of three weeks when tested with sulfuric acid and sodium nitrite or with paradimethylamido-benzaldehyde. There was, however, a noticeable amount of ammonia produced when tested with Nessler's reagent as early as 10 hours after incubation at 30° C.

*Blood agar plates.*—On blood agar plates the colonies have a white center and an irregular periphery. They closely resemble small colonies of *B. subtilis*. A halo-like zone of hemolysis appears immediately about the periphery of the colony. The alkali liberated during the growth of organism is most probably responsible for the hemolysis.

**Reaction Inhibitory to the Growth of *B. lactimorbi*.**—The reaction inhibitory to the growth of the organism was determined both on solid and in liquid media. Eight strains were examined. On nutrient agar the most luxuriant growth took place when the reaction was alkaline (−3.0, −2.5, −2.0 per cent). With an increase in the acidity of the agar there was scanty growth. The organisms were swollen, of indefinite outline, or in the act of sporing. Growth ceased entirely at +2.0 per cent. Similar results were obtained when the organism was grown in sugar-free broths of various degrees of alkalinity and acidity. Altho there was observed a slight variation as regards the tolerance of the organism for acid or alkali according to the strain examined, it can be laid down that on the whole the organism grows best in an alkaline medium. Some varieties refused to multiply in broth containing more than 10 to 15 c.c. normal HCl acid per liter. Other strains grow well in 15 c.c. Scum or distinct pellicle formation was observed only when the organism was grown in an alkaline broth.



**Relation to Free Oxygen.**—The feeble growth in the depth of the glucose-agar stab and the absence of growth in the closed arm of the fermentation tubes indicate that the organism is aerobic in nature. Five strains were grown for six days at 30° C. in a Novy jar containing H<sub>2</sub>. No growth was observed at the end of that period. The organism can, therefore, be designated as an obligatory aerobe.

#### EXPERIMENTAL FEEDING OF DOGS.

Dogs were used exclusively in the feeding experiments to be reported. Prior to the feeding with sporogenous gelatin cultures of *B. lactimorbi* all animals were put under careful observation eleven days or more. During this period loss or gain in weight, disposition, appetite, and character of feces were noted, and served as a check on symptoms which followed the feeding of the organism.

**Dog 5.**—Fox-bull-terrier. Male, well nourished, healthy. Weight on the 1st, 5th, and 11th days, respectively: 10 kgs. 260 gm., 10 kgs. 200 gm., 10 kgs. 200 gm. Gentle, timid, excellent appetite, excretions normal. Protocol follows:

November 22, 1908: Received 80 c.c. of a four-day sporogenous gelatin culture of *B. lactimorbi* ("Alva"). General condition good. Weight, 10 kgs. 200 gms. Excretions soft. Disposition normal. Appetite good.

November 23, 24, 25: Dog received 50 to 80 c.c. of sporogenous culture of *B. lactimorbi*. No change from the normal.

November 26: Lethargic. Trembles. Hind legs particularly involved in the tremors. Looks rather emaciated. Retches repeatedly. Vomitus consists of stringy "cotton" mucus. In a drowsy condition. Refuses to leave the box. Not vicious. Attentive to call. Fifty c.c. of a sporogenous gelatin culture of *B. lactimorbi* ("Alva") were mixt with the food and placed in the box. Appetite fair. Retching ceased. Feces small in amount and semi-solid.

November 27: General condition about the same as on the preceding day. Both eyes inflamed. Left sclerotic deeply injected. Disposition unchanged. Scant feces are semi-solid in nature. Refuses to eat or drink.

November 28: General condition unchanged. Easily fatigued. Walked slowly up two flights of stairs, but refused to walk the third flight. Very thin and emaciated. Mucus discharge from the eyes and nose. Coughs and retches after slightest exertion. Attentive. Pricks up ears at the call of whistle. Prefers to be left alone. Refuses to eat. Drinks freely of water. Constipated. After great effort passed 5-10 gm. of a semi-solid brown colored feces which contained *B. lactimorbi* in great numbers. Weighs 7 kgs. 820 gm., i. e., lost 2 kgs. 380 gm. in six days.

November 29: The animal was found dead at ten o'clock A. M., and in my absence was examined by Dr. Jordan and Dr. Harris to whom I am indebted for the following report of the gross, microscopic, and bacteriological findings.

"Animal not entirely cold. Rigor mortis present. Extremely emaciated. External evidences of having passed thin brownish feces. No subcutaneous fat. Muscles of thorax deep red. No odor of acetone.

*Abdominal cavity.*—No peritonitis, no excess of fluid, no visceral or parietal ecchymoses. Omentum almost devoid of fat. Intestines throughout empty of feces, caliber small. *Small intestine* excised and opened through the entire length. There

was much sticky yellow (bile-stained) mucus everywhere but particularly in the duodenum and upper one-third of the gut; also numerous flat and round worms. On washing, the duodenal portion was much injected, and the mucosa edematous; there were no definite hemorrhages in the mucous membrane, and no erosions. There was no definite folliculitis in the tract. Injected areas were found here and there widely separated. Peyer's patches were not involved. They lay below the general level of the mucosa, and were of a brownish-gray color. *Large intestine and appendix:* Altho outwardly not showing any evidences of an abnormal state, the whole tract appeared somewhat edematous, but more striking was the degree and extent of the injection of the mucosa, which in one or two places actually constituted a hemorrhage. No folliculitis could be demonstrated. The appendix presented a similar injected condition. The colon was preserved in Kaiserling's solution. The *mesenteric glands* were moderately enlarged. The *liver* could not be said to be enlarged. Its color was a deep maroon. It was friable. On section it dripped much blood and altho the lobules could be seen there was some degree of cloudy swelling. *Spleen* not enlarged, pale, and of normal consistence. Section disclosed no abnormalities. *Kidneys:* Both looked alike; enlarged and congested, of a deep maroon color. On section much blood oozed out and there was a considerable degree of cloudy swelling. Interlobular vessels distinct, Malpighian tufts seen with difficulty. *Adrenal glands* pale in color.

*Thorax.*—The lungs showed a small degree of collapse on opening of the chest. They presented a mottled appearance, deep red areas amid lighter ones; and in these deep red areas grayish areas about 2 mm. could be seen scattered about; these latter were soft and on section appeared purulent. Evidently a condition of bronchopneumonia. There were signs of an early pleurisy in the left cavity of the thorax. On section of both lungs numerous areas of a deep red color were seen, as well as mucopurulent contents in the smaller bronchi. *Heart and pericardium:* No excess of fluid in the pericardial sac. No pericarditis. No ecchymoses. Right side of the heart was distended, left collapsed. One or two pin-point ecchymoses at the roots of the great vessels. Color of the organ darker than normal. On section the muscle looked a trifle darker than normal and showed a mild degree of cloudy swelling. Its consistence was softer than normal. The anterior cusp of the tricuspid valve gave evidence of an old healed endocarditis. On one of the cusps (ventral side) of the pulmonary artery was a subendocardial hemorrhage of an irregular size, linear in extent, about  $\frac{1}{2}$  by 3 mm.

Tissue samples were preserved for study in 10 per cent formalin and in 95 per cent alcohol. The paraffin sections of the tissues were stained with hematoxylin and eosin.

**MICROSCOPIC.** *Liver.*—Hyperemic. Otherwise normal.

*Colon.*—Normal.

*Small intestine.*—Normal.

*Heart.*—Some bundles of fibers appear to be normal. Others are fibrillated and contain many small fat droplets.

*Kidney.*—A moderate grade of acute glomerulonephritis, the parenchymatous changes being chiefly in the convoluted tubules, altho others of the loops of Henle and collecting tubules are affected. In places there is complete necrosis of the epithelium.

*Spleen.*—Greatly hyperemic, even the Malpighian bodies are to a considerable extent infiltrated with blood. Some brown pigment scattered about the section within the cells for the most part.

*Cerebrum.*—Appears normal.

*Lung.*—Section taken through a superficial purulent area is filled with round and

polynuclear leukocytes. Aveolar capillaries hyperemic. The pleura is only slightly inflamed.

**BACTERIOLOGICAL.**—Cultures were made on agar from the heart, bronchopneumonic areas in the left lung, left pleura, liver, bile, kidney, mucus of the small intestine.

*B. lactimorbi* was isolated from liver, spleen, and intestinal mucus."

**Dog 6.**—Black and white. Bitch, alert, slender build. Weight constant during period of "control" observation. Affectionate, appetite normal, feces normal. From November 11 to December 27, 1908, received daily an average of 135 c.c. of a four-day sporogenous gelatin culture of *B. lactimorbi* ("Ph1-Bile"). Dog never showed the least sign of being ill. Early in January the animal was etherized and immediately examined. Since the organs revealed no gross anatomical lesions they were not examined microscopically. The serum possessed a high agglutinating power for the "Alva" strain as can be seen from Table 1.

TABLE 1.

STRAIN	SERUM	AGGLUTINATION OBSERVED AFTER 24 HOURS						Control
		1:10	1:50	1:100	1:500	1:1,000	1:5,000	
"Alva"	Normal Dog	+	—	—	—	—	—	—
"Alva"	Dog 6	+	+	+	+	+	—	—
"Alva"	Normal Human	—	—	—	—	—	—	—

Unfortunately a 0.9 per cent NaCl suspension of the "Bile" strain with which the dog had been fed agglutinated spontaneously and it was necessary to test the agglutinating action on various other strains of *B. lactimorbi*. The following table gives the result of the experiment:

TABLE 2.

SERUM OF DOG 6	AGGLUTINATION AFTER 24 HOURS						Control
	1:10	1:50	1:100	1:500	1:1,000	1:5,000	
Strain "Alva"	+	+	+	+	+	—	—
Strain "Ontario"	+	+	—	—	—	—	—
Strain "H. J."	+	+	—	—	—	—	—

Normal dog's serum possessed no agglutinating power on *B. lactimorbi* in a greater dilution than 1:10; normal human serum possessed no agglutinating action whatever. We have here, possibly, an instance of an organism not sufficiently pathogenic to cause disease but able to stimulate the production of specific agglutinins.

**Dog 7.**—Brown, sleek, well-nourished bitch. Weight: 9 kgs. 800 gm. Constant during the period preliminary to the feeding of *B. lactimorbi*. Excellent appetite, affectionate. From November 11 to December 28, 1908, received daily 180 c.c. of a four-day sporogenous culture of *B. lactimorbi* (Strain: "Brain"). Shortened protocol follows:

November 12–December 13, 1908: Nothing abnormal. Increase in weight due to pregnancy.

December 14: Gave birth to three pups. Remained in the box with young. Shivers occasionally. Vicious when persons come near box. Refuses to eat. Feces well formed.

December 15: Back to normal condition. Remained so until December 26.

December 26: Refuses to leave box. Face looks drawn. Slight tremors of the hind limbs. Wags tail and pricks up ears at call of whistle. Coaxed out of box once. On the whole in a drowsy, stuporous condition. Prefers to be left alone. Closes eyes while in a standing position as if going to sleep. Vicious when other dogs come near the box. Attentive to the young. Feces partly solid, partly soft. Feces contain little mucus.

December 27: Feces very soft. Animal in normal condition.

December 28: Feces semi-solid and blood stained. General condition normal.

From December 28, 1908, to February 2, 1909, dog remained in good health. The dog was killed with ether on February 3 (six weeks after giving birth to the pups and about one month after the last feeding with gelatin cultures of *B. lactimorbi*).

AUTOPSY.—Well-nourished bitch. Weighs 10 kilos. Has bad case of mange. Complete physiological atrophy of the mammary glands. No external abnormalities.

*Abdominal, pleural, and pericardial cavities* normal. *Heart and lungs* normal. Lungs are slightly congested. *Peribronchial lymph glands* are not enlarged, slightly anthracotic. *Mesenteric and retroperitoneal lymph glands* are not enlarged.

*Gastro-intestinal tract, spleen, and kidneys* normal.

*Liver*.—About normal in size. Mottled, yellow and white areas alternating with reddish areas. The liver is exceedingly yellow. There are several patches of irregular outline (2 by 3 cm. and smaller) which are distinctly white. The liver has a fatty appearance and touch. The lobules are well marked. Liver parenchyma exceedingly friable. Cut surface of the liver has a yellowish appearance. Small congested areas noted on the external surface are found to be only subserous. Gall-bladder is full of a thick golden-colored bile. No concretions in the gall-bladder.

*Urinary and generative tract* normal.

ANATOMICAL DIAGNOSIS.—Mange. Slight fatty infiltration of the liver.

MICROSCOPIC.—Celloidin sections. Hematoxylin and eosin.

*Myocardium* normal.

*Lungs*.—Alveolar capillaries highly congested and contain numerous polymorphonuclear leukocytes. Otherwise normal.

*Liver*.—Naked-eye examination of the stained sections shows a strikingly mottled appearance. On microscopic examination it is found that the mottled appearance is due to the fact that the central portions of the lobule take on a fainter hematoxylin and eosin stain than does the periphery of the lobule. The cells near the central vein are slightly infiltrated with fat. Sections of the tissue taken from the periphery of the organ show some cells having the typical signet-ring appearance. Here there is also subcapsular congestion. In some places the protoplasm of the cells is entirely replaced by fat globules. There is no congestion of the intralobular vessels. Marked leukocytosis. Scattered throughout the section of the liver are small foci or aggregates of round cells. There are approximately 20 to 30 cells of the lymphoid type to such a focus. The nuclei of these round cells stain intensely with hematoxylin. In some of the foci can be seen epitheloid cells and polymorphonuclear leukocytes. These foci are not limited to any particular part of the section, appearing at one time near the central vein, at another time at the periphery of the lobule. The interlobular connective tissue is normal in amount. The numerous foci of round cells together with the pronounced leukocytosis suggested the possibility of *B. lactimorbi* within the foci. Sections of the liver were stained for bacteria. No organisms were found within the foci.

*Spleen* normal.

*Kidneys*.—Blood vessels of the kidneys are highly congested. Glomeruli are somewhat contracted. Renal epithelium normal. There are no casts.

BACTERIOLOGY.—Cultures made from the liver, spleen, and kidney were found sterile. *B. lactimorbi* could not be isolated from the intestinal mucus.

**Pups**.—As recorded in the preceding protocol, Dog 7 gave birth to three pups. One of the pups was found dead the morning after the delivery. An autopsy was immediately made.

Extensive hemorrhage into the anterior abdominal wall about the umbilicus. The hemorrhagic area was 3 cm. long and 2 cm. wide. Condition of general anasarca with hydrothorax and great amount of clear watery ascitic fluid. The pup was still-born as shown by the floating test. The organs were slightly pink in color and flabby. Beyond this no anatomical lesions were found. Cultures made from the liver were sterile. *B. lactimorbi* was, however, isolated; from the peritoneal transudate and from the kidney. Unfortunately no tissues were kept for microscopic examination.

The remaining two pups were in a poor condition, in part due, no doubt, to the fact that they were poorly nourished by the mother. After the mother had been autopsied they were immediately put on a rich diet to increase their general condition. Unfortunately one of the pups died from injuries received by falling from its cage. The condition of the other pup gradually improved and feeding was begun on February 22 with milk and gelatin cultures of the "Alva" strain. The pup received daily about 50 c.c. of a five-day milk or gelatin culture of *B. lactimorbi*. Feeding continued uninterruptedly until May 1. The pup never showed any signs of being sick. On March 25 some blood was withdrawn and its agglutinating power determined for various strains of *B. lactimorbi*, with the following result:

After 34 hrs.	agglutination complete	in 1:500	for "Alva" strain.
"	"	"	in 1:200 for "Brain" strain.
"	"	"	in 1:50 for "Ontario" strain.
"	"	No agglutination	in 1:10 for "H. Park" strain.
"	"	"	in 1:10 for "A. W." strain.

AUTOPSY (May 22). **Dog 8**.—Age: 5 months. Weight: 4,950 gm. Growth markedly stunted. Subcutaneous fat abundant. Peritoneal, pleural, and pericardial cavities contain the normal amount of clear fluid. The internal organs with the exception of the liver showed no pathological changes. The liver was soft and friable. Had a slight pink color. Aside from a dull yellowish pink coloration cut surface showed no gross anatomical lesions. Outline of lobules not to be made out. In consistence the liver was soft. Large patches of the capsule of the liver were easily torn from the surface of the liver.

Organs were sterile. *B. lactimorbi* could not be isolated from the gastrointestinal tract. Pieces of the various organs and tissues were fixed in Zenker's, imbedded in celloidin, and the section stained with hematoxylin and eosin.

MICROSCOPIC.—*Kidneys, lungs and myocardium* normal.

*Liver*.—Contains very little blood. There is no sharp line of separation between individual lobules. Normal lobular structure with the trabeculae of liver cells alternating with the intralobular capillaries is absent. The lobule seems to be made up entirely of large highly granular fatty liver cells with rather indistinct nuclei. The swollen



liver cells have led to an almost entire obliteration of the intralobular capillaries. Connective tissue normal in amount.

**Dogs 9 and 10.**—These dogs were fed daily with large quantities of *B. lactimorbi*. On an average each dog received 180 c.c. of a four-day sporogenous gelatin culture of the organism. Dog 9 received cultures of the "H. J." strain isolated by Jordan and Harris from a human being dead from milksickness; Dog 10 received gelatin cultures of the organism isolated from the spleen of Dog 5. In spite of these large quantities of the organism fed daily to the dogs for a period of three weeks, neither dog ever showed the slightest degree of illness. Experimentation was therefore discontinued and the dogs were autopsied. No gross anatomical lesions were found.

#### DISCUSSION AND SUMMARY.

It is remarkable that *B. lactimorbi* with its pronounced and characteristic morphological and tinctorial features and its wide distribution in nature should have escaped notice for so long a time. C. Günther<sup>1</sup> describes a bacillus isolated by him from water which possessed motility, liquefied gelatin, and formed drumstick spores. A microphotograph of the sporing organism closely resembles *B. lactimorbi*. I have been unable to get the original article in which Günther described the organism in greater detail. Another organism isolated by Grether<sup>2</sup> from canal water, and to which Günther refers as a closely allied form, has cultural features which very closely resemble those of *B. lactimorbi*. This organism repeatedly isolated by Grether from canal water is about 1:5  $\mu$  long, grows well at room temperature and at 28° C. but not at 37° C., possesses drumstick spores which in stained preparation appear oval, forms yellow moist growth on potato accompanied by a discoloration of the potato, grows only in the bulb of the fermentation tube containing slightly alkaline dextrose broth, but is not motile, nor does it cause liquefaction of gelatin. The similarity of the organisms described by Günther and Grether to *B. lactimorbi* is apparent and the morphology and the cultural features as far as they are given strongly suggest that they may be related organisms. Owing to its polymorphic tendency the growth of *B. lactimorbi* on agar plates and on agar slants may and often does resemble the luxuriant, spreading growth of *B. subtilis*, and on this account has undoubtedly heretofore been mistaken for *B. subtilis* or for organisms of the subtilis group.

The close association of *B. lactimorbi* with only certain plants as

<sup>1</sup> *Einführung in das Studium der Bacteriologie*, 1898, p. 575; and plate IV, Fig. 23.

<sup>2</sup> *Archiv f. Hyg.*, 1896, 27, p. 226.

noted above is of general bacteriological interest, and certainly invites further investigation. The contrast between plates poured from growth of timothy hay and alfalfa is most striking; the latter contain numerous colonies of *B. lactimorbi*, the former contain practically nothing but the large spreading colonies of *B. subtilis*. Of the five weeds gathered in the Pecos Valley, New Mexico, where the disease was first studied by Jordan and Harris, four (*Portulaca pilosa*, *Solanum elaeagnifolium*, *Gutierrezia sphaerocephala*, and *Bigelovia Rusbyi*) yielded strains of *B. lactimorbi*. *Senecio filifolius* collected from the same region contained no organism in any respect similar to *B. lactimorbi*. The search for the organism on clover, hominy, bran, and silage likewise yielded negative results. From dried alfalfa the organism was isolated with great ease. The samples of alfalfa were collected for the most part from areas where milksickness has never been known to occur and from farms which supply Chicago and Milwaukee with certified milk.

Some of the strains show minor cultural differences. "Por.," "A. I.," and "Sol." caused coagulation of milk after 72 hours. The reaction of the milk is alkaline. Resolution of the precipitated casein was complete on the eighth day, the milk having at the end of this period a dirty brownish turbid appearance. Growth of strains, "Gutierrezia," "Brain," and "Bigelovia," on plain agar slants resemble very much growth of *Strept. pyogenes* on the same medium. Aside from these differences the cultural and biochemical features of the saprophytic strains and of the "pathogenic" strains isolated from man and animal dead or dying from milksickness were identical.

Of the six dogs inoculated two (Dog 5 and Dog 7) showed in a slight degree the symptoms observed in milksickness: stupor, coma, muscular weakness, retching, and loss of appetite. It will be recalled that Dog 5 died as a result of illness. At the autopsy there was found bronchopneumonic condition of the lungs. This constituted the most conspicuous macroscopic and microscopic lesion. Cloudy swelling of the liver and edema of the colon with marked injection of the mucosa which in one or two places actually constituted hemorrhage were the other gross anatomical findings. Microscopically the liver is described as "hyperemic, otherwise normal," and the colon and remaining tissues with exception of lungs

are described as being "normal." It is highly probable that the cause of death in this instance was the bronchopneumonia. If we take these points into consideration it is difficult to understand how *B. lactimorbi* isolated from a normal liver, normal spleen, and from normal intestinal tract of this animal should have been the cause of the disease and the primary cause of death when the organism was not present in the distinct bronchopneumonic patches. It is possible that *B. lactimorbi* caused the bronchopneumonic condition and then disappeared from the lesions as there were no bacteria in the areas.

The results of experimentation with Dog 7 are more decisive. This dog was taken sick with symptoms slightly simulating the symptoms observed in milksickness, but recovered. At the autopsy the animal showed a highly friable liver with slight fatty infiltration. Microscopic examination of the liver revealed (aside from slight fatty infiltration) numerous healed focal necroses. Moreover, this dog gave birth to three pups one of which was still-born. *B. lactimorbi* was isolated from the peritoneal transudate and from the kidney of this pup.

Dog 8 (pup) on the other hand never showed the slightest degree of illness in spite of the fact that the dog received large quantities of the organism daily for a very long period of time. Altho no illness resulted there was a pronounced retardation of growth. The serum of the dog, likewise, possessed a rather high agglutinating power for several strains of *B. lactimorbi*. At autopsy the liver was found very soft and friable. In all other respects the dog was normal. Histologically the tissues were normal with exception of the liver. The liver showed parenchymatous degeneration and beginning fatty degeneration.

Dogs 6, 9, and 10 never exhibited the slightest degree of illness. Organs and tissues were found normal. It is true that the agglutinating power of the serum of Dog 6 lay between 1:1,000 and 1:5,000 for the "Alva" strain. It is, however, entirely probable that the body would react even against the most saprophytic organism when given in large quantities with the production of specific agglutinins which would cause sedimentation even in high dilution. The same may also explain the high agglutinating power of the serum of Dog 8 (pup).

The preceding experiments are, I believe, far from being decisive in establishing *B. lactimorbi* as the etiological factor in the production of milksickness. The organism either loses its pathogenicity very rapidly when grown on artificial media; or the virulence of the organism is dependent on what may be termed a symbiotic life or existence on certain plants. It is possible that its growth on such plants as the rayless goldenrod (*Bigelovia*), white snake root (*Eupatorium ageratoides*) is responsible for its pathogenic power. Both of these plants have been thought to be the cause of milksickness, and bacteriological examination of the rayless goldenrod from the Pecos Valley, New Mexico, demonstrated that at least half of the organisms found associated with this plant consisted of *B. lactimorbi*. I have had no opportunity of examining bacteriologically *Eupatorium ageratoides*. This plant grows abundantly about Joliet, Ill., where milksickness is more or less prevalent throughout the year. Whether the bacillus when growing on certain plants experiences an increase in its pathogenic power or whether it produces from the plant substance a toxic material which is responsible for the symptoms of the disease can only be offered as conjectures. On the other hand, it is remarkable that if *B. lactimorbi* be the cause of milksickness, it should have so wide a distribution in milksick and non-milksick regions. It has also been apparent that loss of virulence occurs after a time in the races of the organism isolated, and that no feeding experiments with several strains of *B. lactimorbi* have so far yielded uniformly any clear-cut pathological picture of the disease. Up to the present no method has been devised which will increase the virulence of the organism.

## STUDIES ON THE CHEMISTRY OF ANAPHYLAXIS (II).\*†

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IN a previous paper<sup>1</sup> were reported observations on the effects of tryptic digestion on the anaphylactic properties of bovine serum. It was found that as the proportion of coagulable nitrogen decreased, the power of the digestion mixture to intoxicate guinea-pigs sensitized with bovine serum, and to sensitize normal pigs to bovine serum, decreased in quite the same ratio. The results, as far as the experiments were recorded in that paper, were as follows: After 21 days' digestion of serum, when the coagulable nitrogen had been reduced to 8 per cent of the total nitrogen, the toxicity of the serum had been so nearly destroyed that 5 c.c. intraperitoneal doses caused no symptoms in sensitized pigs. It sensitized, however, in doses of 0.004 c.c. but not in doses of 0.0004 c.c., as contrasted with normal serum of which the minimal sensitizing dose is 0.0001 to 0.00001 c.c. The specificity of the reaction does not seem to be impaired by the digestion. A sample taken after 59 days' digestion contained 4.3 per cent of coagulable nitrogen, and its minimum sensitizing dose was 0.02 c.c. The toxicity was so reduced that 5 c.c. intraperitoneally, or 1 c.c. by intracardiac injection produced no symptoms in pigs sensitized with bovine serum; however, the same doses were toxic to pigs sensitized with the digestion mixture. Further digestion until the 129th day reduced the coagulable nitrogen to 2.5 per cent of the total nitrogen, and increased the minimum sensitizing dose to 0.1 c.c., without impairing the specificity.

Since that time the digestion has been continued, with the following results: After digestion from February 28 to November 1, nine months, the serum ("Digestion Mixture V") still gives a very faint turbidity on heating and acidifying, too small in amount to determine the nitrogen by the Kjeldahl method, and no distinct flocculi or other evidence of coagulable protein. The biuret reaction is still present but very faint. Injection into guinea-pigs gave the following results:

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† This work has been aided by the Memorial Institute for Infectious Diseases.

<sup>1</sup> *Jour. Infect. Dis.*, 1908, 5, p. 449.



TABLE 1.

First Injection	Interval	Second Injection	Results
1. 0.1 c.c. bovine serum.....	23 days	5 c.c. digest. mixt. V*	No symptoms
2. 1.0 " digest. mixt. V.....	20 "	5 " " " "	" "
3. 0.1 " bovine serum.....	23 "	2 " " " "	(Intracardiac) slight symptoms
4. 0.1 " " " ".....	23 "	1 " " " "	(Intracardiac) doubtful symptoms
5. 0.1 " " " ".....	28 "	10 " " " "	No symptoms
6. 0.02 " digest. mixt. V.....	20 "	4 " bovine serum	" "
7. 0.05 " " " ".....	" "	4 " " " "	" "
8. 0.1 " " " ".....	" "	4 " " " "	" "
9. 0.5 " " " ".....	" "	4 " " " "	No definite symptoms
10. 1.0 " " " ".....	" "	4 " " " "	Severe symptoms (recovered)
11. 1.0 " " " ".....	" "	5 " " " "	" "

\* Unless otherwise specified injections were made intraperitoneally.

Gave 3 c.c. bovine serum to each of Nos. 3, 4, and 5, 24 hours after the second injection, and all died with typical symptoms within one hour, showing absence of protective action by the digestion mixture.

The sensitizing power of the serum has, therefore, been so greatly reduced by nine months' digestion that the minimum sensitizing dose is 1 c.c., in contrast to the sensitizing dose of normal serum, which is one hundred-thousandth of a cubic centimeter, or less. The intoxicating power has been so far reduced that it cannot be detected by intracardiac injections of 1 to 2 c.c. or by intraperitoneal doses of 5 to 10 c.c. Larger doses cannot be used, as the digestion mixture possesses a certain degree of toxicity for normal pigs, which limits the size of the dose that may be used.

Digestion was continued until January 7 (314 days), and another sample taken. This sample gives a slight turbidity on neutralizing and heating, but no distinct biuret reaction. The results of injection into guinea-pigs were as follows:

TABLE 2.

First Injection	Interval	Second Injection	Results
1. 0.5 c.c. digest. mixt. VI....	17 days	3 c.c. bovine serum	Doubtful symptoms
2. 1.0 " " " ".....	17 "	" " " " "	Died in 3 hours
3. 2.0 " " " ".....	19 "	" " " " "	" " 1/2 "
4. 2.0 " " " ".....	19 "	" " " " "	Slight symptoms
5. 2.0 " " " ".....	20 "	" " " " "	" "
6. 4.0 " " " ".....	17 "	" " " " "	Severe "
7. 6.0 " " " ".....	17 "	" " " " "	Moderate "
8. 10.0 " " " ".....	17 "	" " " " "	Severe "
9. 2.0 " " " ".....	17 "	10 " digest. mixt. VI	Doubtful " *
10. 8.0 " " " ".....	17 "	" " " " "	" "
11. Normal pig.....	" "	" " " " "	Same as 9 and 10
12. " " " ".....	" "	" " " " "	" "
13. 0.2 c.c. bovine serum.....	20 days	" " " " "	Doubtful symptoms
14. 0.1 " " " ".....	22 "	" " " " "	" "
15. 2.0 " digest. mixt. VI....	" "	" " " " "	" "
16. 2.0 " " " ".....	" "	" " " " "	" "
17. 0.1 " bovine serum.....	" "	2 " " " "	(Intracardiac) doubtful symptoms
18. 0.1 " " " ".....	" "	" " " " "	" "

\* The pigs injected with the digestion mixture all became more or less sick, but the symptoms were not the same as in typical anaphylaxis, and normal pigs were fully as much affected as sensitized pigs.

Nos. 14, 17, and 18 were each given 4 c.c. bovine serum 24 hours after the second injection, and all three died with typical symptoms within 30 minutes.

Therefore the further digestion of this serum has not reduced the sensitizing power appreciably, the minimum dose still being about 1 c.c., altho the serum now has no distinct coagulability or biuret reaction. The digestion mixture is of itself quite toxic, but no more so for sensitized than for normal pigs, the symptoms resembling somewhat anaphylactic symptoms at first, but coming on without the usual period of incubation, and persisting 24 hours or more. As pigs that had been sensitized to bovine serum were killed by injection of bovine serum subsequent to injection of the digestion mixture, it is probable that symptoms produced by this digested serum are due to its own toxicity and not to any anaphylactic intoxication, for in the latter case the animals would have been refractory to bovine serum.

After removal of the sample used in the previous experiment (314 days' digestion), the remaining 200 c.c. of fluid was filtered free from precipitated substances, a fresh sample of trypsin (0.2 gm.) was added, and digestion was continued at 37° until July 6, 1909, a total period of over 16 months. It still gives a slight turbidity on neutralizing and heating, apparently due to traces of coagulable protein that cannot be removed by prolonged digestion, but no biuret reaction. When injected into guinea-pigs in doses of 1 to 5 c.c. it sensitizes them so that they react slightly, but typically, to bovine serum injected three weeks later, the most marked reaction being obtained in the pigs that have received the 5 c.c. doses; in no case was the reaction at all severe.

From these experiments with bovine serum it would seem that the anaphylaxis reaction depends upon some constituent of the serum that decreases in amount *pari passu* with the coagulable protein, but which persists after over a year's digestion with fresh quantities of trypsin. As the minimum dose of serum which will sensitize at this stage is about 1 or 2 c.c., and as the sensitizing dose of a pure protein (egg albumin) has been found to be as small as one-twentieth of a millionth of a gram, it is possible, and indeed probable, that the sensitizing power of the digested serum is due to the presence of coagulable protein which is still present in too small amounts to be detected by the heat test, which test is not adequate to detect any quantity as minute as this might be. The fact that the sensitizing and intoxicating power decrease together may be considered as

further evidence in support of the view maintained by Rosenau and Anderson, that the sensitizing and intoxicating principles of serum are one and the same, and not different proteins. However, it would be perfectly possible that two different proteins, one having sensitizing and one intoxicating properties, might be destroyed at approximately equal rates by digestion with trypsin.

An interesting phenomenon, which perhaps is of significance, is that when the serum has been digested to a point where it has but little toxicity for guinea-pigs sensitized with normal bovine serum, it is much more toxic for guinea-pigs that have been sensitized with the digested serum.<sup>1</sup> Apparently a special sensitization exists here, the guinea-pigs injected with digested serum being sensitized for undigested serum proteins by the still unaltered serum protein molecules, while at the same time they are sensitized specifically by some of the partially digested molecules for similarly altered molecules which are present in the digested serum and absent in the fresh serum. As subsequent experiments, to be described below, indicate that ordinary albumoses and peptones are not capable of either sensitizing or intoxicating guinea-pigs, in the sense of the anaphylactic reaction, it is probable that the molecules concerned in the above phenomenon are molecules of serum protein that have not been disintegrated quite as far as to the stage of albumoses. Obermayer and Pick observed a similar condition in their studies of the effects of tryptic digestion of serum upon its power to produce and to react with precipitins, for they found that the serum of rabbits immunized with such digested serum reacted only with the homologous digested serum, and not with normal serum.

#### DIGESTION OF EGG ALBUMIN BY TRYPSIN AND PEPSIN.

Experiments on the effects of tryptic digestion on crystallized egg albumin were not very successful, because the chloroform, which needs be added to prevent putrefaction, coagulates the albumin and thus destroys its sensitizing and intoxicating effects, which, as previously shown, are only produced when the protein is injected in a soluble form. No other antiseptic than chloroform seemed available, for it is the antiseptic that can be most readily removed from

<sup>1</sup> See Tables 13 and 14 of the previous paper.

the solution before injection, and, furthermore, egg albumin is coagulated by nearly all other antiseptics as much as or more than it is by chloroform. The following protocol will show the result of an experiment with tryptic digestion with egg albumin:

Dissolved 5 gm. crystallized egg albumin in 200 c.c. of 0.4 per cent sodium carbonate solution, and added 0.5 gm. active pancreatin and 5 c.c. chloroform. After standing four days at 37° the mixture showed a heavy precipitate, and the filtered fluid gave only a slight turbidity on being neutralized, boiled, and acidified with acetic acid. Injected this unfiltered mixture into pigs that had been sensitized 13 days previously with five milligrams of egg albumin each, with the following results:

1. Injected 5 c.c. of digestion mixture, but animal showed no distinct symptoms. Twenty-four hours later injected 0.15 gm. egg albumin, and the animal showed typical but not severe anaphylaxis symptoms.

2. Injected 10 c.c. of digestion mixture, and obtained slight but apparently typical symptoms. Forty-eight hours later gave 0.1 gm. egg albumin, which produced slight but typical symptoms.

3. Injected 10 c.c. of digestion mixture into a guinea-pig, which showed typical but not severe symptoms. Twenty hours later 0.15 gm. egg albumin was injected which killed the animal in 20 minutes.

For purpose of control a similar mixture was prepared with the pancreatin left out, and it was found to be of a jelly-like consistence (alkali albuminate) after standing five days in the incubator. Injected into pigs previously sensitized with albumin, the filtrate from this mixture produced much the same effects as recorded in the three previous experiments.

After the digestion mixture had been four days in the incubator it was left at room temperature for 13 days, and then a part was used for sensitization of four pigs, the rest kept in the ice box for later injections. The results of these injections were as follows:

1. Injected 2 c.c. digestion mixture, and 18 days later gave 0.1 gm. egg albumin. No distinct symptoms until after one hour, and then a slight degree of sickness and paralysis of the hind legs appeared, which persisted for 24 hours.

2. Injected 0.1 c.c. digestion mixture, and 18 days later gave 0.1 gm. egg albumin. Same effects as in No. 1, but less severe.

3. Injected 1 c.c. digestion mixture, and 19 days later gave 10 c.c. of the same mixture. Slight but typical symptoms after 15 minutes.

4. Injected 0.01 c.c. digestion mixture, and 19 days later gave 10 c.c. of same mixture. Slight but typical symptoms after 15 minutes.

From these experiments it is evident that the sodium carbonate and chloroform alter the egg albumin so much that any effect produced by the trypsin would be entirely masked. However, as shown by another series of experiments, digestion of 5 per cent egg albumin solution by trypsin for two months, when the filtrate gave only a faint biuret reaction, and no turbidity on neutralization and heating, rendered it so inert that even 10 c.c. doses did not sensitize guinea-

pigs in the least to egg albumin, and it had no intoxicating effect whatever upon pigs sensitized to egg albumin even when given by intracardiac injections. Apparently crystallized egg albumin is less resistant to tryptic digestion than is bovine serum, possibly because the former is deprived of antibodies in the process of crystallization, for antibodies are usually found to be contained in the globulin fraction of native proteins.

Peptic digestion of egg albumin was much more successful, for the antiseptic action of the hydrochloric acid was sufficient to prevent putrefaction without the addition of chloroform. The digestion mixture consisted of 5 gm. crystallized egg albumin and 0.5 gm. active pepsin (from pig) in 100 c.c. *n*/10 HCl. As Michaelis<sup>1</sup> found that the power of protein to react with precipitins is destroyed very quickly by peptic digestion (in 40 minutes in the case of serum), it was anticipated that a similarly rapid effect would be produced on the power of egg albumin to sensitize and to intoxicate, but such was not the case. At intervals 5 c.c. of the digestion mixture was pipetted off, and 5 c.c. *n*/10 NaOH added to neutralize it; the 10 c.c. of neutral mixture was then injected into pigs sensitized with egg albumin between two and three weeks previously, with the following results:

TABLE 3.

Duration of Digestion	Result of Injection into Sensitized Pigs	Remarks
1. 5 min.....	Death in 12 min.	.....
2. 30 ".....	" " 40 "	.....
3. 65 ".....	Severe symptoms	Recovered
4. 2 hours.....	Death in 40 min.	.....
5. 4 ".....	" " 85 "	.....
6. 7 ".....	" " 1½ hours	.....
7. 12 ".....	Severe symptoms	Recovered
8. 13 ".....	Died after 1½ hours	Died in night
9. 24 ".....	" in " "	*
10. 30 ".....	Slight symptoms	.....
11. 48 ".....	Doubtful "	†Symptoms apparently due to toxicity of digestion mixture rather than to anaphylaxis
12. 72 ".....	" "	.....

\* Digestion mixture now (24 hours) yields but a very slight precipitate of acid albumin upon neutralization, but still considerable coagulable protein is present. This is, however, too small in amount to be determined by Kjeldahl's method in a 5 c.c. sample, there being not over 1 or 2 mg. of coagulable nitrogen.

† Solution after 48 hours' digestion showed only a mere trace of coagulable protein, but heavy biure reaction.

<sup>1</sup> *Ztschr. klin. Med.*, 1905, 56, p. 417.



This experiment indicates that peptic digestion of egg albumin destroys its power to intoxicate sensitized guinea-pigs only when it has destroyed practically all coagulable protein, indicating that this property of proteins is less readily modified than is the property of reacting with precipitins, according to the observations of Michaelis. This author also found that peptic digestion of serum until the coagulable protein was all removed, destroyed its power of acting as a precipitogen, showing that this property of protein, like its sensitizing property, is more resistant than its property of being precipitated by specific precipitins. In experiments recorded in the next table it is shown that the sensitizing power of egg albumin persists even after all positive evidence of coagulable protein has been lost, suggesting that the sensitizing agent is even more resistant than the precipitogens, possibly because larger amounts of precipitins must be present to be demonstrable. Pick and Yamanouchi,<sup>1</sup> failing to destroy the anaphylactic properties of serum by digestion 15 minutes with pepsin-HCl, conclude that this is evidence that the precipitin reaction and the anaphylaxis reaction are dependent on different substances, a conclusion which Michaelis<sup>2</sup> shows is unwarranted. As a matter of fact the experiments reported herewith show that the resistance of the sensitizing principle to pepsin is not very different from the resistance of the precipitogens as determined by Michaelis, if we take into consideration that a considerable amount of precipitogen must be present in a solution to be demonstrable by its production of precipitins, while sensitization may be accomplished by the merest traces of protein.

There is a little question concerning the influence of the pepsin in these experiments, for it was found that the conversion of albumin into acid-albumin had a slight effect upon its power to intoxicate sensitized guinea-pigs, as shown by the following experiment:

One gram crystallized egg albumin was dissolved in 20 c.c. water, and 3 c.c. of 20 per cent HCl added. This caused a heavy precipitate, and the mixture was left standing at room temperature over night. It was then filtered, washed, and the precipitate was suspended in 20 c.c. of water and dissolved in a minimum amount of NaOH. This solution was then injected in doses of from 3 to 5 c.c. into four guinea-pigs that had been sensitized two weeks previously, causing the death of one, severe symptoms in another,

<sup>1</sup> *Ztschr. f. Immunitätsforschung*, 1909, 1, p. 676.

<sup>2</sup> *Ibid.*, 1909, 2, p. 294.

but only slight symptoms in the other two. The two last pigs were injected 48 hours later with egg albumin, one reacting severely, the other but slightly.

Four animals were also sensitized with the acid albumin solution, in the following doses: No. 1, 5 c.c.; No. 2, 2 c.c.; No. 3, 1 c.c.; No. 4, 0.1 c.c. Each was injected with 0.15 gm. albumin 10 days later with the following results: No. 1 showed slight symptoms; No. 2 became severely sick but recovered; Nos. 3 and 4 died each in 35 minutes.

Apparently, then, acid albumin is somewhat less effective in intoxicating pigs sensitized to natural albumin than is the natural albumin itself, but it by no means loses either its intoxicating or sensitizing effects. Converting egg albumin into alkali albuminate, however, seems to have a much more marked influence upon its anaphylactic activity. Egg albumin converted into alkali albuminate, precipitated with acetic acid, washed, and redissolved in alkali, caused in 0.1 gm. doses absolutely no symptoms in guinea-pigs that had been sensitized to egg albumin, and left them just as sensitive to egg albumin as control pigs.

Even prolonged digestion with pepsin, beyond the time of disappearance of recognizable coagulable protein and acid albumin, does not entirely destroy the sensitizing power of egg albumin, as shown by the following table:

TABLE 4.

Duration of Digestion	Result of Injection of Egg Albumin	Condition of Digestion Mixture Used for Sensitizing
1. 24 hours.....	Death in 20 min.	Some coagulable protein
2. 48 ".....	" " " "	Trace of " "
3. 72 ".....	" " 15 "	" " " "
4. 4 days.....	" " 15 "	Faint turbidity on heating
5. 5 ".....	Slight symptoms	" " " "
6. 6 ".....	Moderate " "	" " " "
7. 8 ".....	" " " "	Trace of " "
8. 12 ".....	Slight " "	Doubtful " "
9. 18 ".....	No " "	" " " "
10. 26 ".....	Moderate " "	No " " " "
11. 36 ".....	Died in 40 min.*	" " " "
12. 36 ".....	Doubtful symptoms	" " " "

\* This exceptional result may be due to a sensitized pig having been accidentally used.

The results of the foregoing experiments with digestion mixtures, indicating that the anaphylaxis reaction depends either upon whole protein molecules, or at least on molecules but slightly altered from the native coagulable state, did not make it probable that the isolated cleavage products of the proteins would be found to be actively anaphylactic, nevertheless a number of preparations of albumoses

and peptones were made from egg albumin and tested on guinea-pigs, as shown by the following summaries:<sup>1</sup>

A. Albumose was prepared from raw egg white by digestion with pepsin-HCl, removal of coagulable proteins, precipitation by saturation with ammonium sulfate dialysis, and carefully purified by twice reprecipitating. In doses of 0.25 gm. and over this caused the animals to become slightly sick, but doses as high as 0.5 gm. did not usually produce serious effects. Several guinea-pigs were injected with from 0.05 to 0.5 gm. of this albumose, and after 14 days given 0.1 gm. doses of egg albumin, or 0.3 gm. doses of albumose. A few of the animals showed slight atypical symptoms, but no conclusive evidence of anaphylaxis was obtained. Guinea-pigs sensitized with egg albumin were found not susceptible to the albumose, and injection of albumose left them still susceptible to egg albumin.

B. Albumose prepared from coagulated egg white in the same way as above, was equally inert, neither sensitizing pigs to egg albumin or albumose, nor intoxicating pigs that had been previously given sensitizing doses of albumin or albumose. As with albumose A, pigs sensitized to egg albumin, and injected after two weeks with the albumose preparations, were still quite as susceptible to a second dose of egg albumin as if they had not received the albumose injection, indicating that the albumose from egg albumin causes no reaction whatever in animals sensitized to egg albumin.

C. A series of fractions of the products of digestion of raw egg white by an extract of fresh pig pancreas was prepared, including the ordinary peptones, and several fractions of the crystalline and non-crystalline products. None of these had any power of sensitizing guinea-pigs to itself or to egg albumin, or of intoxicating guinea-pigs that had been sensitized to egg albumin, or of rendering such sensitized animals refractory to egg albumin.

The results of the foregoing experiments leave little room for doubt that the anaphylaxis reaction can be produced only by intact protein molecules, or at least by molecules of proteins less removed from the whole proteins than are the ordinary albumoses and peptones. That some of the first products of protein cleavage may have sensitizing powers for the whole protein molecules is suggested by the following experiments:

A. Dissolved 3 gm. crystallized egg albumin in 150 c.c. water, heated it to 100° for 45 minutes, added a drop of dilute acetic acid, filtered, and washed. Ground the coagulum in a mortar, added 60 c.c. of 0.5 per cent sodium carbonate solution, 0.4 gm. pancreatin, and 2 c.c. chloroform. Kept at 38° C. After varying periods took homogenous samples, filtered, and injected the filtrate into guinea-pigs, with the following results:

1. Forty-eight hours' digestion. Filtrate from a 10 c.c. sample injected into guinea-pig previously sensitized with egg albumin. Animal became slightly sick, but symptoms were not altogether typical of anaphylaxis; apparently they were due rather to the toxicity of the mixture, as 48 hours later an injection of 0.15 gm. egg albumin caused death in 35 minutes.

<sup>1</sup> Most of these preparations were made in the Sheffield Biological Laboratory of Yale University to the staff of which I am indebted for much advice and assistance.

A second 5 c.c. sample was injected into a normal guinea-pig, and 15 days later this animal was given 0.1 gm. egg albumin which caused severe, typical, but not fatal symptoms.

2. Five days' digestion. Ten c.c. sample caused no distinct symptoms in pig sensitized to egg albumin; and the animal reacted typically but not fatally to egg albumin injected 4 hours later.

A normal guinea-pig given 5 c.c. of this digestion mixture reacted slightly but typically when injected with egg albumin 14 days later.

3. Seven days' digestion. No distinct symptoms caused by 10 c.c. injected into a sensitized guinea-pig, and injection of egg albumin 4 hours later caused the death of the animal in 30 minutes.

4. Twelve days' digestion. Five c.c. sensitized a guinea-pig so that when injected with egg albumin 17 days later it became severely and typically sick.

5. Twenty-one days' digestion. Two guinea-pigs receiving 5 c.c. of this material showed no symptoms on subsequent injection with egg albumin.

**B.** Dissolved 2 gm. crystallized egg albumin in 150 c.c. water, and heated at 100° for one hour. Filtered, and injected the clear, water-like filtrate, after concentration to 10 c.c., into a normal guinea-pig. Twenty-two days later the guinea-pig reacted typically, altho not severely, to an injection of egg albumin.

Ground up the coagulum, added 40 c.c.  $n/10$  HCl and 0.1 gm. pepsin, and kept at 37°. At intervals of 24, 48, 100, and 150 hours withdrew 5 c.c. samples, neutralized with 5 c.c.  $n/10$  NaOH, and injected the mixture without filtration into normal guinea-pigs. All four pigs reacted typically when injected with egg albumin 15 days later, none fatally, but the pig receiving the 24-hour sample became extremely sick. When injected into pigs sensitized with egg albumin this digestion mixture produced no definite symptoms.

From these experiments it would seem that some of the products of hydrolysis of coagulated egg albumin have to a slight extent the power of sensitizing to egg albumin. Either the amount of these sensitizing substances is exceedingly minute, or else they do not sensitize very effectively to the unaltered egg albumin, for the sensitized guinea-pigs never reacted fatally and only occasionally was the reaction severe. Corresponding to this feeble sensitizing power, the intoxicating effect of the products of digestion of boiled egg albumin upon sensitized pigs is practically nothing.

#### SPECIFICITY OF THE PROTEINS OF EGGS.

Among the various proteins that have been isolated from eggs, besides the albumin, are ovomucoid and ovovitellin. The results of experiments with ovomucoid are shown in Table 5, p. 516.

From these experiments a number of interesting facts may be learned. In the first place the fact that ovomucoid intoxicates severely, sometimes fatally, guinea-pigs sensitized with ovomucoid,

is most conclusive proof of the opinion supported in the previous article, that the effects of heat upon the substances concerned in the anaphylaxis reaction with serum and egg albumin are due solely to the coagulation of the proteins. Ovomucoid is prepared by heating solutions of egg white to boiling to coagulate all coagulable proteins; the filtrate, usually many liters in amount, is evaporated over the flame or on the water bath to a small volume, and the ovomucoid precipitated with alcohol, redissolved in water, and reprecipitated. During this process the ovomucoid is subjected to a temperature at or near the boiling point for some hours, and also is precipitated with

TABLE 5.

First Injection	Interval	Second Injection	Results
1. 0.0025 gm. ovomucoid.....	10 days	0.25 gm. ovomucoid	Died in 7 hours
2. 0.0005 " " " " " " " "	" "	0.25 " "	Severe typical symptoms
3. 0.0005 " " " " " " " "	" "	0.25 " "	Slight " "
4. 0.0005 " " " " " " " "	" "	0.20 " "	Severe " "
5. 0.0005 " " " " " " " "	" "	0.1 " egg albumin	No distinct symptoms
6. 0.012 " " " " " " " "	22 " "	0.15 " "	Slight symptoms*
7. 0.012 " " " " " " " "	" "	0.1 " ovomucoid	Died in 35 minutes
8. 0.005 " " " " " " " "	16 " "	0.1 " "	Slight symptoms
9. 0.0005 " " " " " " " "	" "	0.1 " "	" "
10. 0.00005 " " " " " " " "	" "	0.1 " "	" "
11. 0.005 " " " " " " " "	" "	0.15 " egg albumin	Very slight symptoms
12. 0.0005 " " " " " " " "	" "	0.15 " "	" " "
13. 0.00005 " " " " " " " "	" "	0.15 " "	" " "
14. 0.005 " egg albumin.....	30 " "	0.2 " ovomucoid	No symptoms
15. 0.005 " " " " " " " "	" "	0.2 " "	" " †
16. 0.005 " ovovitellin.....	20 " "	0.25 " "	" " †

\* Twenty-four hours later received 0.1 gm. ovomucoid and became moderately but typically sick.

† Twenty-four hours later injection of 0.1 gm. egg albumin into Nos. 14 and 15 caused death in less than 15 minutes.

alcohol several times, yet in spite of this treatment it is able to cause typical anaphylaxis effects. Unquestionably the persistence of anaphylactic activity in ovomucoid in spite of all this heating and precipitating, depends upon the fact that ovomucoid does not lose its solubility in water because of these manipulations.

Also, the experiments on cross-sensitization of crystallized egg albumin and ovomucoid show that each of these substances is specific for itself, altho both come from a common source, the hen's egg. This is an important point in view of the unsettled state of our information concerning species specificity, as indicated by the precipitin test. The commonly accepted statement is that precipitins are specific for the species that furnishes the antigen, but that they will not distinguish between two different proteins from the same species.



There is, however, some evidence that precipitins can be obtained that will distinguish at least quantitatively between two different proteins from the same species, but the evidence is not altogether harmonious. The results here recorded with the ovomucoid are so important that the work will be extended later when a larger amount of ovomucoid has been collected.<sup>1</sup> It may be mentioned in this connection that Rosenau and Anderson<sup>2</sup> were able to sensitize guinea-pigs with guinea-pig placenta, a fact which, in conjunction with the ovomucoid results, suggests that the specificity exhibited by the anaphylaxis reaction may be somewhat different from the specificity shown by the precipitin reaction.

Similar indications of specificity were obtained in a few experiments with ovovitellin from the hen's egg, as follows:

TABLE 6.

First Injection	Interval	Second Injection	Results
1. 0.005 gm. ovovitellin.....	20 days	0.25 gm. ovomucoid	No symptoms
2. 0.005 " " .....	" "	0.1 " egg albumin	Slight
3. 0.005 " " .....	" "	ovovitellin*	Death, one hour
4. 0.005 " " .....	" "	" "	Moderate symptoms
5. 0.05 " " .....	" "	0.15 gm. egg albumin	Slight
6. 0.05 " " .....	" "	ovovitellin	Died in 45 minutes

\* This preparation of ovovitellin, for which I am indebted to Dr. T. B. Osborne of New Haven, is not very soluble in weak alkalis on account of frequent extraction with alcohol for the purpose of removing lecithin. Consequently, the exact dosage was not readily determined, and in these experiments where the dosage is not specified 10 c.c. of 0.2 per cent NaOH solution saturated with ovovitellin was used, probably containing from 0.05 to 0.1 gm. of protein.

#### RELATION OF CLEAVAGE PRODUCTS OF PROTEINS TO THE ANAPHYLAXIS REACTION.

Recently Biedl and Kraus<sup>3</sup> have reported studies on anaphylaxis in dogs, which led them to conclusions which seem surprising to one accustomed to working with guinea-pigs. Dogs do not react violently to a second dose of serum, and have not generally been considered suitable for anaphylaxis experiments, but Biedl and Kraus find that there is a marked fall of blood pressure when foreign serum is injected into sensitized dogs, which is not observed when the injection is made into normal dogs. This fall of blood pressure seems to depend upon a primary peripheral vaso-dilatation, and these authors con-

<sup>1</sup> For the preparation used in the above experiments I am indebted to Mr. M. S. Fine of the Sheffield Biological Laboratory.

<sup>2</sup> *Bulletin 45*, Hygienic Laboratory, June, 1908.

<sup>3</sup> *Wien. klin. Wchnschr.*, 1909, 22, p. 363.

sider that it is the chief phenomenon in the anaphylaxis reaction. Accompanying the fall of blood pressure is a decrease in the coagulability of the blood, and as these two phenomena are also produced by "peptone" they sought for a relationship between anaphylaxis and peptone intoxication. This they believe they have established by the following observations:

The symptoms following the injection of Witte's peptone into dogs in intravenous doses of 0.3 to 0.03 gm. per kilo are the same in all respects as the symptoms of anaphylaxis in dogs; if Witte's peptone is given to dogs sensitized to serum (horse or bovine) they react in the same way to it as do normal dogs, but are thereby made insusceptible to a subsequent injection of serum; in other words, a sensitized dog reacting to peptone becomes refractory to serum, exactly as if the second dose had been serum rather than peptone. On the other hand, animals given two doses of serum, and reacting after the second, may then either react or fail to react to a subsequent dose of Witte's peptone. They quote the old observations of Pick and Spiro that proteoses (Witte's "peptone" consists chiefly of proteoses) are not themselves toxic and that the observed toxicity is due to contaminating substances, being unfamiliar with the later work of Underhill<sup>1</sup> which seems to establish that proteoses are themselves toxic. They therefore conclude that the anaphylactic reaction is induced by some poison which is related to or identical with the poisonous element of Witte's peptone, a conclusion quite similar to that propounded some time ago by Vaughan, with whose work Biedl and Kraus seem to be unacquainted.

Richet<sup>2</sup> has criticized the article of Biedl and Kraus at some length, having himself observed in his actinotoxin experiments the fall in blood pressure, but not the loss of coagulability. He agrees with them that the action is not upon the heart, but doubts the reliability of their conclusion that the vaso-dilatation is not a secondary phenomenon due to disturbance of the central nervous system. As he points out, the fall of blood pressure of itself is not sufficient to account for all the features of anaphylaxis, for in the first place an equally great fall of blood pressure produced by amyl nitrite or other

<sup>1</sup> *Amer. Jour. Physiol.*, 1903, 9, p. 345

<sup>2</sup> *Presse Méd.*, 1909, 17, p. 249.

means does not cause any such profound effects; and secondly, there are many irritative and paralytic phenomena which are obviously entirely independent of the lowered blood pressure. Furthermore, the anaphylactic reaction is specific, while the peptone reaction is not, and in addition, the quantity of "peptone" necessary to produce symptoms is far greater than the quantity of protein necessary to call forth violent manifestations of anaphylaxis.

It should also be suggested that the results obtained by observing changes in the blood pressure in dogs are by no means comparable with results obtained with guinea-pigs, upon which most of the work so far reported has been done, since in these animals the symptoms are entirely different from the symptoms in the dog, and much more closely resemble the effects seen in man.<sup>1</sup> Indeed, even in the guinea-pig the symptom complex varies distinctly and strikingly with the protein used, so that it is possible to tell with some assurance whether a reaction is being produced with egg white, which causes highly irritative effects, or with serum, which causes more paralytic symptoms. One may also comment on the drawing of conclusions from results obtained with a substance of so uncertain and unknown composition as Witte's peptone.

In any event, the crucial point of Biedl and Kraus's proof of the relationship of anaphylaxis to peptone intoxication, namely, that injection of peptone into dogs sensitized to serum renders them refractory to serum, is not in harmony with results obtained in experiments which have been carried out with guinea-pigs, in conjunction with the studies of the relation of cleavage products of proteins to anaphylaxis reported in this and the previous paper. Numerous observations have been made showing that guinea-pigs sensitized to serum and egg albumin are not rendered refractory to the homologous protein by injection of either the entire products of digestion of these proteins by pepsin or trypsin, or by the isolated fractions of the digestion products. Furthermore, the various

<sup>1</sup> As having an important bearing on the results of Biedl and Kraus, may be quoted from Underhill's article the following statements as to the influence of species upon the effects of proteoses: "The organism of the dog is particularly susceptible to the effects of intravenous injections of the products of proteolysis. In the cat the characteristic symptoms are evoked somewhat less readily, larger doses being necessary to produce comparable results. The rabbit, on the other hand, is extremely resistant, and, except in rare cases, fails to respond in so far as the phenomena involving the blood are concerned. . . . Although commercial 'propeptone' preparations are fatal to guinea-pigs they fail to render the blood incoagulable except when injected rapidly into fasting animals."

cleavage products and digestion mixtures do not produce in either normal or sensitized guinea-pigs the symptoms characteristic of anaphylaxis. Even when given in lethal doses the symptoms are entirely dissimilar, and death occurs after many hours, even days, unlike the sharp violent anaphylaxis reaction.

The lack of influence of products of protein digestion upon anaphylaxis is shown in the following table:

TABLE 7.

Sensitizing Sub-stance	First Intoxicating Dose	Result	Second In-toxicating Dose	Result
1. Egg albumin...	Albumose "A"	No symptoms	Egg albumin	Death
2. " " "	" " "A"	" "	" "	" "
3. " " "	" " "B"	" "	" "	" "
4. " " "	" " "B"	" "	" "	" "
5. " " "	Peptone (tryptic)	" "	" "	" "
6. " " "	" "	" "	" "	" "
7. " " "	" (peptic)	" "	" "	Slight symptoms
8. " " "	" "	" "	" "	Severe
9. " " "	" "	" "	" "	Death
10. " " "	" "	" "	" "	" "
11. " " "	Crystalline products of tryptic digestion	" "	" "	" "
12. " " "	Same as No. 11	" "	" "	" "
13. " " "	Last fraction of tryptic digestion	" "	" "	" "
14. " " "	Same as No. 13	" "	" "	" "
15. " " "	Entire products of tryptic digestion of coagulated egg albumin	" "	" "	" "
16. " " "	Same as No. 15	" "	" "	Severe symptoms
17. " " "	Same as No. 15	" "	" "	Death
18. " " "	Digestion of raw egg white (tryptic)	" "	" "	Moderate symptoms
19. " " "	Same as No. 18	Doubtful	" "	" "
20. " " "	Same as No. 18	No	" "	Death
21. " " "	Same as No. 18	" "	" "	" "
22. Bovine serum	Digestion mixture V	" "	Bovine serum	Moderate symptoms
23. " " "	" "	" "	" "	Death
24. " " "	" "	" "	" "	" "
25. " " "	" " V1	" "	" "	" "
26. " " "	" " "	" "	" "	" "
27. " " "	" " "	" "	" "	" "
28. " " "	" " VIA	Doubtful	" "	Moderate symptoms
29. " " "	" " "	" "	" "	" "

NOTE.—The digestion products mentioned in the above table may be described briefly as follows: Albumose "A" is the mixture of albumoses obtained from coagulated egg white by peptic digestion, removal of coagulable protein, precipitation by saturation with ammonium sulfate; dialysed, and purified by being twice precipitated with ammonium sulfate and dialyzed. Albumose "B" is a similar product from peptic digestion of raw egg white. Peptone "tryptic" and "peptic" are the alcohol precipitable fractions from tryptic and peptic digestion of raw egg white, after removal of albumose, purified by dialysis. After removal of the peptone from the tryptic digestion mixture, several crops of crystalline material were obtained (Experiments 11 and 12), and after these were removed a non-crystalline mass was left (Experiments 13 and 14). In Experiments 15 to 21 egg albumin was digested with trypsin until free from coagulable protein, and the entire mixture injected. Digestion mixtures V, V1, VIA are the entire products of tryptic digestion of bovine serum for various periods of time, as described previously in this article.

## SUMMARY.

It is extremely difficult to digest serum entirely free from coagulable protein by trypsin, a specimen digested over 16 months still showing possible traces of coagulable material. Such serum in doses of 1 c.c. and over still sensitizes guinea-pigs to the homologous protein, altho not fatally. The intoxicating power disappears before the sensitizing power, corresponding to the much smaller dose of the latter which is necessary to produce effects. Digestion of serum does not affect its specificity, but partially digested serum is more toxic to guinea-pigs sensitized with the digested serum than to pigs sensitized with normal serum. Trypsin destroys the sensitizing and intoxicating power of crystallized egg albumin much more readily than it affects serum, presumably because this purified albumin is free from globulins, which are the proteins of serum and tissues that are most resistant to trypsin. Pepsin-HCl digestion of egg albumin destroys its sensitizing and intoxicating properties very slowly, the former persisting to a slight degree even after coagulable protein cannot be longer demonstrated. Conversion of egg albumin into acid albumin somewhat impairs, but by no means destroys, its power to sensitize guinea-pigs to egg albumin and to intoxicate pigs that have been sensitized with egg albumin. Conversion of egg albumin into alkali albumin, however, renders it entirely inert to pigs sensitized with egg albumin.

The products of digestion of egg white by pepsin or trypsin which can be separated by the ordinary methods, i. e., albumoses, peptones, polypeptids, crystallizable amino-acids, etc., have no power to sensitize or intoxicate guinea-pigs, whether used in conjunction with themselves or with undigested egg white.

These experiments indicate that proteins cannot be decomposed much if any beyond the coagulable form without losing their anaphylactic properties. For the anaphylaxis reaction we must have intact protein molecules in soluble form. Possibly there exists a stage immediately between the entire protein molecule and the ordinary proto- and deutero-proteoses, with which anaphylaxis can be produced, for coagulated proteins digested with either pepsin or trypsin show at certain stages a slight power to sensitize animals to egg albumin, but the power is very slight.



Preliminary experiments indicate that ovomucoid (and possibly also ovovitellin) has a specific anaphylactic action, reacting only with itself and not with other proteins of egg white. This form of specificity, i. e., a protein causing reaction with its own kind and not with other kinds of protein from the same species of animal, has not been conclusively demonstrated with the precipitin reaction, and is of so much importance in the larger problems of biological specificity that it will be investigated further. As ovomucoid causes fatal anaphylaxis reactions after having been heated for several hours at or near the boiling point, and after being precipitated and reprecipitated with alcohol, we have conclusive evidence that moist heat affects the anaphylactic property of proteins only when it renders the proteins insoluble.

The evidence adduced by Biedl and Kraus that in dogs the anaphylactic reaction is produced by substances related to Witte's peptone finds no confirmation in experiments performed with guinea-pigs. They observed that Witte's "peptone" administered to dogs sensitized to horse serum rendered the dogs refractory to subsequent injections of horse serum, as shown by blood pressure measurements; but in guinea-pigs entire digestion mixtures, or isolated fractions of digestion products, did not render sensitized pigs refractory to the homologous proteins. Apparently there are marked differences in the anaphylaxis reaction in different animals, and there seem to be some differences in the reactions produced by different proteins.

## COCCIDIOIDAL GRANULOMA AND BLASTOMYCOSIS IN THE CENTRAL NERVOUS SYSTEM.\*

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DURING several years recently there have been reported numerous cases of two closely related groups of an unusual form of chronic infectious disease belonging pathologically to the granulomata and resembling in many features tuberculosis. I refer to coccidioidal granuloma and blastomycosis. The cases of coccidioidal granuloma have been described mostly in California, and by far the greater proportion of the cases of blastomycosis have been studied in Chicago. The most striking biological feature of these organisms is that in the tissues they always appear in the one case as sporulating organisms (coccidioidal), in the other as budding fungi (blastomycetes); while in artificial culture they have an entirely different appearance, both organisms invariably growing as molds.

**Coccidioidal Granuloma.**—In 1892 Posadas and Wernike<sup>1</sup> in Buenos Ayres first reported a case of the disease now called coccidioidal granuloma, the organism of which is named *Oidium coccidioides* by Ophüls of San Francisco, who has carefully studied more of these cases than any other observer. As many as 17 or 18 cases of this disease have been collected.<sup>2</sup> It is a chronic infectious disease having a great resemblance clinically and pathologically to systemic tuberculosis. The lesions, which are principally abscesses and nodules, have been found in practically all the organs of the body. The course of the disease varies from three months to a number of years. It seems to be invariably fatal, altho the individual lesions may heal. In the majority of the cases the primary focus of infection apparently is the lungs. Other cases are seen in which the first lesions appear in the skin or other superficial structures. But these later invariably show pulmonary lesions. In those cases beginning in the lungs, some later develop skin lesions while others do not.

In coccidioidal granuloma a typical spherical, double contoured organism is always found. It varies in size up to  $30\ \mu$  or even more in diameter, and in the tissues always multiplies by endogenous sporulation, and budding forms (blastomycetes) have not been seen. However, in the pus from abscesses, typical budding organisms have been observed.<sup>3</sup> In artificial culture external to the body it always grows as a mold, an abundant growth of mycelia being produced.

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<sup>1</sup> *Jour. de Microgr.*, 1891, 15, p. 14.

<sup>2</sup> Brown, *Jour. Amer. Med. Assoc.*, 1907, 48, p. 743.

<sup>3</sup> *Ibid.*

The first and the most extensive work on the morphology of this organism was by Rixford and Gilchrist.<sup>1</sup>

The geographical distribution of the observed cases of this disease is remarkable. Outside of the one case reported from South America all the cases have been observed in California or have lived in California, and the great majority of them have resided at one time or another in the San Joachin Valley.

Of these 18 cases only two (possibly three) have been reported as showing lesions of the central nervous system.

**Systemic Blastomycosis.**—In 1894 Gilchrist described the first case of blastomycetic infection of the skin, and gave it the name of "Blastomycetic Dermatitis." Since that time as many as 100 or more cases of blastomycetic infection of the skin having uniform clinical and pathological characteristics have been reported. At least two such cases have been observed and studied in Nashville by Dr. Litterer, and one of these he has reported.<sup>2</sup>

In the same year, 1894, in Germany, Busse and Buschke<sup>3</sup> reported a case of systemic infection with an organism which is recognized as being practically identical with the parasite in blastomycetic dermatitis. In America the first systemic case of blastomycosis was reported in 1892 by Walker and Montgomery in Chicago.<sup>4</sup> Since that time and including the case of Busse and Buschke and one case by Curtis,<sup>5</sup> in Europe, there have been reported 22 cases of undoubted systemic or generalized infection with the blastomycetes. (Montgomery and Ormsby also report five additional cases which probably belong to the same category, but which have not been so proved by autopsy.<sup>6</sup>) Most of these cases have been fatal, but at least two have recovered. These systemic cases are very similar in their clinical aspect and gross pathology to those mentioned above as coccidioidal granuloma.

It is a remarkable fact that a large proportion of the cases of blastomycosis, both cutaneous and systemic, have been observed in Chicago.

Of the 22 systemic cases, five have been reported as having lesions of the central nervous system, three having focal lesions in the substance of the cerebrum and cerebellum, one a destructive process of the spinal cord, and one, the case of Curtis, having a clinical meningitis.

The parasites found in the purely cutaneous affection and in the systemic blastomycoses are identical, altho certain variations are seen in the characteristics of the organisms in the different cases. They are always seen in the tissues and in pus from abscesses as spherical, sometimes oval organisms, 8 to 20  $\mu$  in diameter, having the characteristic refractive capsule giving them the so-called double contoured appearance. Budding forms are constantly seen in the tissues and in the pus from the abscesses. In artificial culture it grows as a mold with typical mycelia and conidial spores.

The organisms of coccidioidal granuloma and of blastomycosis are very similar, both in their appearance in the affected tissues and in artificial culture. The most striking and apparently essential difference is in the fact that in the coccidioidal granuloma the forms seen

<sup>1</sup> *Johns Hopkins Hospital Reports*, 1896, 1, p. 290.

<sup>2</sup> *South. Med. Jour.*, 1908, 1, p. 294.

<sup>3</sup> Buschke *Die Blastomykose*, 1902.

<sup>4</sup> *Jour. Amer. Med. Assoc.*, 1902, 38, p. 867.

<sup>5</sup> *Ann. de l'Inst. Pasteur*, 1846, 10, p. 449.

<sup>6</sup> *Arch. Int. Med.*, 1908, 2, p. 1.

in the tissues are always multiplying by sporulation, and never by budding, while in the blastomycetic cases the multiplication is typically by budding. Whether this morphological difference is unequivocal evidence that the two organisms belong to two distinct classes is open to question for the following reasons: 1. In the pus from abscesses in some of the coccidioidal cases typical budding forms are seen, altho in the tissues these forms are always absent. 2. The marked similarity in the characteristics of their growths upon artificial media. 3. In one case of systemic blastomycosis LeCount<sup>1</sup> observed in a large tubercle of the cerebellum undoubted sporulation forms, tho in adjacent portions of the same lesion only the budding forms could be found. 4. In one of the earlier cases of systemic blastomycosis, a study of which was reported by Otis and myself in 1903,<sup>2</sup> the lesions in the internal organs of an inoculated guinea-pig showed the typical sporulation forms identical in appearance with those seen in the coccidioidal cases.

These facts indicate an exceedingly close relationship between the organisms in these two classes of disease, and it is possible that the two organisms are simply variations of an identical form. Montgomery and Ormsby<sup>3</sup> express the opinion that in a given case there are probably usually present more than one strain of these parasites which accounts for the so-called pleomorphism seen in the cultures. In regard to the relation of these two organisms Jordan<sup>4</sup> says: "A singular and more fatal disease of a similar character has been observed in the San Joaquin Valley on the Pacific Slope of the United States. Although the organism found was at first thought to be a protozoan, and the disease is still termed 'coccidioidal granuloma,' there is now no doubt that the parasite concerned is a blastomyces or 'oidium.'"

Ophüls<sup>5</sup> says of the relation and classification of these organisms, "The resemblances between lesions and parasites in blastomycetic dermatitis and those in coccidioidal granuloma seem close enough to my idea to classify them together, perhaps after Ricketts'<sup>6</sup> suggestion, as Oidiomycosis."

Hektoen says with reference to this point: "Systemic blastomy-

<sup>1</sup> *Jour. Infect. Dis.*, 1908, 4, p. 187.

<sup>2</sup> *Jour. Amer. Med. Assoc.*, 1903, 41, p. 1075.

<sup>3</sup> *Arch. Int. Med.*, 1908, 2, p. 1.

<sup>4</sup> *General Bacteriology*, Phila., 1908, p. 421.

<sup>5</sup> *Jour. Exper. Med.*, 1905, 6, p. 443.

<sup>6</sup> *Jour. Med. Res.*, 1902, 6, p. 377.

cosis and coccidioidal granuloma are caused by closely related varieties of organisms that Ricketts and Ophüls would place with the *oidia*.”<sup>1</sup>

**Previously Reported Cases of Coccidioidal Lesions in the Central Nervous System.**—1. Ophüls.<sup>2</sup> (Case 2.) German, age 50, laborer. Clinical history not obtainable. Comatose on entrance to hospital. Paresis of muscles of right side of face. Remained comatose or partially so till time of death, which occurred after six days. No record of any skin lesions. Autopsy: “Small scars in apices of both lungs. Pleuritic adhesions. Purulent infiltration and (pseudo) tuberculosis of pericardium at base of heart. Chronic pericarditis with complete obliteration of pericardium. (Pseudo) tuberculosis of meninges, spleen, and both kidneys. Ependymitis chronica nodosa.” The principal changes in the brain were a purulent leptomeningitis at the base of the brain, particularly in the fissure of Sylvius, the pia being infiltrated with pus and containing numerous submiliary sized yellow nodules. Nodules also seen at the bottom of the longitudinal fissure, and in the ependyma.

Microscopically the pia much thickened with many small abscesses, containing fibrin, nuclear fragments, polymorphonuclear leukocytes, and peripherally, lymphocytes, plasma cells, large mononuclear cells, also giant cells of the Langhans type, and others with the nuclei grouped at the center. Other lesions are composed of plasma and other mononuclear cells and giant cells, no polymorphonuclear cells being present. Parasites are present in these abscesses and nodules, often within giant cells. These organisms are empty shells and sporulation forms mostly. Other affected organs show the same parasites. No tubercle bacilli can be found.

2. Ophüls.<sup>3</sup> (Case 7.) Male, age 19. Sick four weeks previous to entering hospital. Remained in hospital about three months, when he died. High remittant fever. Marked rigidity of neck. Kernig’s sign present. At intervals in stupor, and at times delirious. Twelve days before death unequal pupils and complete paralysis of left abductens muscle developed, and next day paralysis of bladder. No cutaneous lesions. Autopsy: Basilar meningitis with many disseminated nodules in Sylvian fissures, very chronic inflammation of spinal pia, thickening three to four mm. in diameter. In thickened pia, many miliary abscesses. Marked softening of the cord. Coccidioidal parasites, adult and sporulation forms, demonstrated in the meninges. In this case there was also present chronic caseation in the adrenals containing tubercle bacilli. No tubercle bacilli in meninges.

#### **Cases of Systemic Blastomycosis with Lesions in the Central Nervous System.**

—1. Curtis.<sup>4</sup> Male, age 20. Multiple swellings rapidly developing on various parts of body. Some of these swellings formed abscesses and ruptured. Great numbers of blastomycetic organisms found. Cultures and animal inoculation successful. Patient died of meningitis of undetermined nature, after about one year.

2. Eisendrath and Ormsby.<sup>5</sup> Polish laborer, age 33. Illness began February, 1904, death, August, 1906. First symptoms were noticed in the chest. In June, 1904,

<sup>1</sup> *Jour. Amer. Med. Assoc.*, 1907, 49, p. 1071.

<sup>2</sup> *Jour. of Exper. Med.*, 1905, 6, p. 443.

<sup>3</sup> *Jour. Amer. Med. Assoc.*, 1905, 45, p. 1201.

<sup>4</sup> *Ann. de l’Inst. Pasteur*, 1896, 10, p. 449.

<sup>5</sup> *Jour. Amer. Med. Assoc.*, 1905, 45, p. 1045. Also LeCount and Myers, *Jour. Infect. Dis.*, 1907, 4, p. 187.



first cutaneous lesion was seen. Entered hospital February, 1905. April, 1905, blastomycetes demonstrated in sputum. Much improved in hospital, leaving in July, 1905. Returned to hospital after two months, with relapse; gibbus of dorsal spine present. Blastomycetes demonstrated in feces. Autopsy: Blastomycotic broncho-pneumonia. Blastomycosis of peribronchial lymph nodes, pleura, the sub-pleural and retropharyngeal tissue, the liver, kidneys, and colon (?), spinal column (dorsal), external spinal dura, cerebellum, joints, skin and subcutaneous tissue. A fistulous opening extends from right pleural cavity to spinal canal at third dorsal vertebra.

A blastomycotic nodule of right lobe of cerebellum, resembling solitary tubercle,  $4.5 \times 3.8$  cm. This nodule shows a large proportion of necrotic tissue in meshes of well stained tissue. Many submiliary abscesses. Masses of granulation tissue within this mass contain numerous small parasites and forms which are evidently sporulating. LeCount in a later report<sup>1</sup> describes a similar but smaller lesion found in the cerebrum of this same patient.

The remarkable feature of this case was the presence of sporulating organisms, altho, except in this particular portion of the lesion where the sporulating forms were seen, the other organisms were all of the budding forms. The lymph node infection is also rather exceptional in the blastomycetic cases, but common in cases of coccidioidal granuloma.

3. Montgomery and Ormsby.<sup>2</sup> (Case 18.) Patient had cough, and large cutaneous and subcutaneous lesions; blastomycetes in sputum and pus; several joints and a number of vertebrae were affected. Blastomycotic lesions were found throughout the body after death; the bodies of several vertebrae were destroyed, and a portion of spinal cord.

4. Krost, Moes, and Stober.<sup>3</sup> Polish laborer, age 42, sick five months; respiratory symptoms first noticed; abscess in back developed after one month; first skin lesion appeared three months after onset. No symptoms of organic nervous lesions. Miliary and nodular blastomycosis of the lungs, kidneys, spleen, cerebrum, pleura, and lymph nodes found post mortem. In cerebrum, cerebellum, prostate, pleura, skin, and elsewhere were softened foci, five small abscesses being found in the cerebrum and two in the cerebellum, with multiple abscesses in the osseous, muscular, and subcutaneous tissues. Blastomycetes demonstrated in the cerebrum and cerebellum as well as in other places. Cultures were obtained from the lesions, also from the blood.

5. In addition to these cases LeCount<sup>4</sup> in a recent paper reports a case with multiple lesions in the cerebrum and cerebellum occurring at the Cook County Hospital, a detailed report of which is expected to appear later.

It will be noted that in the two cases of coccidioidal granuloma that I abstract there was in each case a well defined basilar meningitis, while in none of the cases of systemic blastomycosis in which nervous lesions appeared, with the exception of the case of Curtis in which no post mortem examination was made, was such lesion present, but the

<sup>1</sup> *Jour. Nerv. and Ment. Dis.*, 1909, 36, p. 144.

<sup>2</sup> *Arch. Int. Med.*, 1908, 2, p. 1.

<sup>3</sup> *Jour. Amer. Med. Assoc.*, 1908, 50, p. 184.

<sup>4</sup> *Jour. Ment. and Nerv. Dis.*, 1909, 36, p. 144.

diseased areas were located in the tissues of the cerebrum and cerebellum, and in one case a destructive lesion of the spinal cord is described.

It is possible that if in all cases of systemic blastomycosis and the coccidioidal disease a more careful post mortem examination of the central nervous system were made, a larger number of lesions of the brain and cord might be found.

The case I report is an additional instance of brain lesion occurring in coccidioidal granuloma. In my case it is much regretted that the clinical history is wanting; I was unable to learn anything of the patient's history, only the tissues in question coming into my possession.

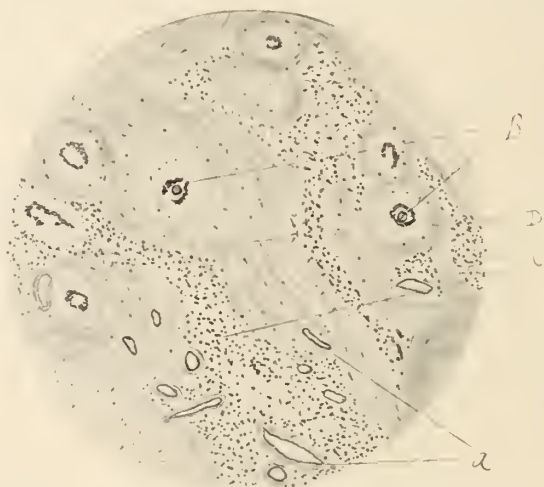


FIG. 1.—Thickened pia under low magnification. A, blood vessels. B, giant cells within round nodules and containing parasites. C, round cells and plasma cells. D, new formed connective tissue.  $\times 50$ .

It is probable that no cultures were ever attempted in this case. Lately it has been learned that the patient had lived at one time in California.

The material examined is from the basal parts, cerebellum and cerebrum, and shows a marked thickening of the pia, which varies from a fraction of a millimeter to four millimeters in thickness. The morbid process affects that portion of the pia extending into the sulci as well as that upon the surface.

Microscopically the thickened tissue is composed of a peculiar granulation tissue having a decided resemblance to tuberculous granu-

lation tissue. In the laboratory the microscopic diagnosis of the tissue at first was "tuberculous meningitis" and until the specific organism had been observed. Careful search for tubercle bacilli in the tissues was made without results. The most striking microscopic feature is the presence of great numbers of submiliary nodules, as shown in Fig. 1. These nodules contain at their centers beautiful large giant cells of the Langhans type, practically every one of which contains one or more of the parasites, the *Oidium coccidioides*. The giant cell is situated at the center of a group of cells corresponding to the so-called "epithelioid cells" among which are scattered a few plasma

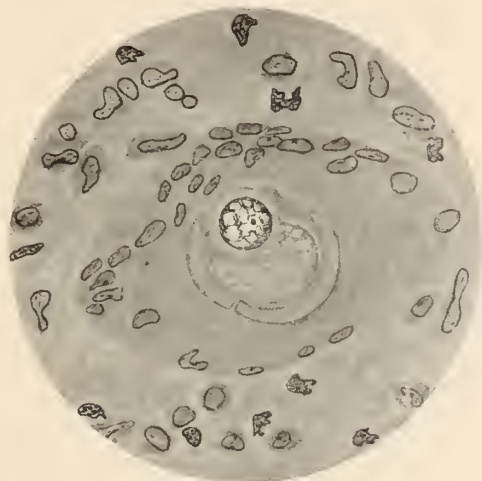


FIG. 2.—Typical large organism with thick hyalin capsule seen within a typical giant cell of the Langhans type.  $\times 450$ .

cells and leukocytes. Surrounding the group of epithelioid cells is seen a thick layer of new formed connective tissue. Around and between these tubercles are extensive areas of tissue filled with plasma cells and small round celled infiltration. The vascular channels are numerous, and many of the arterioles show a definite but not extensive degree of proliferative endarteritis with marked thickening of the subendothelial connective tissue.

In certain of the nodules just described the giant cell is disintegrated or is replaced by a small area of caseation. This is seen particularly in those in which the contained organism has multiplied by sporulation, numbers of smaller organisms occurring within the necrotic area.

The miliary and submiliary abscesses described in most of the lesions of coccidioidal granuloma do not appear here to any great extent. The proportion of polymorphonuclear cells in the cellular infiltration is rather small. There are, however, a few of the typical minute abscesses described by other observers.

#### THE MORPHOLOGY OF THE ORGANISM IN THE TISSUES.

The typical adult parasite within its giant cell is shown in Fig. 2. Practically none of these typical adult forms are seen except within the giant cells of the typical nodule. They are of large size compared to the adult organisms of blastomycosis, some measuring as much as

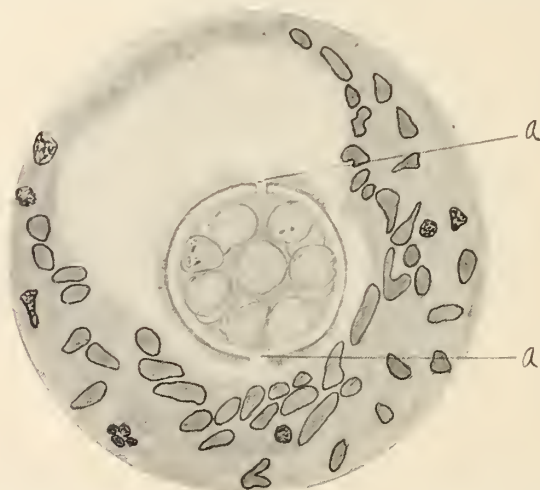


FIG. 3.—Sporulation form within an imperfect giant cell. Capsule ruptured in two places, *a, a*. Capsule covered by short bristles.  $\times 600$ .

approximately  $35\ \mu$  in diameter. They invariably appear perfectly spherical in shape, and their surface consists of a relatively thick capsule which is hyalin, clear, homogeneous, refractive, and not taking any stain by ordinary methods. The cytoplasm of most of them is deeply staining with hematoxylin and contains various sized spaces resembling vacuoles. Some of these large forms have at their surface a corona of radiating prickles or bristles which are described by Rixford and Gilchrist and by Ophüls as appearing typically on the surface of the sporulating form. Many of the sporulating forms as well as the large non-sporulating forms in our case have this appearance as shown in Fig. 3.

In some of the large forms and many of the smaller ones the deeply stained cytoplasm is absent, and the organism looks like an empty

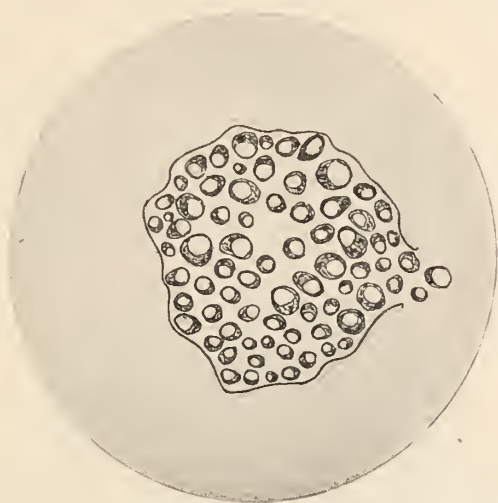


FIG. 4.—A sporulation form containing a large number of small spores within a wrinkled capsule. The spores are escaping through a rent in the capsule. This form is seen only in the minute abscesses.  $\times 700$ .

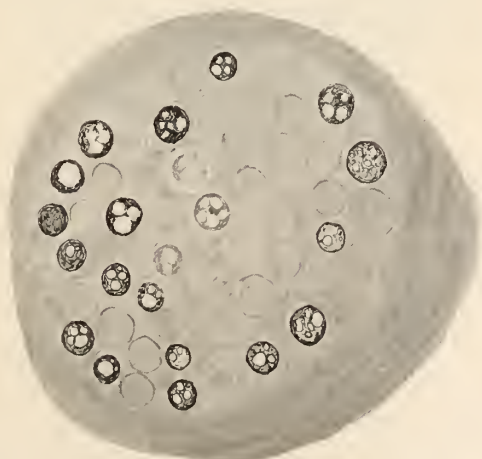


FIG. 5.—Large group of developing spores surrounded by a thick layer of homogenous materia.  $\times 500$ .

spherical capsule. Probably these are capable under favorable conditions of developing into the form taking the stain. Many of the spores and especially the smaller forms which have just emerged



as spores from the parent organism have this appearance. However, many of the clear forms seem to have lost their vitality and have become wrinkled or collapsed so as to have a semi-lunar outline.

#### SPORULATION.

In the sections many forms and groups of the organism are seen which can leave no doubt that reproduction is by sporulation. In harmony with the description of sporulation by Rixford and Gilchrist the division of the cytoplasm evidently takes place by successive division into two, four, eight, sixteen, or larger number of parts. Numer-

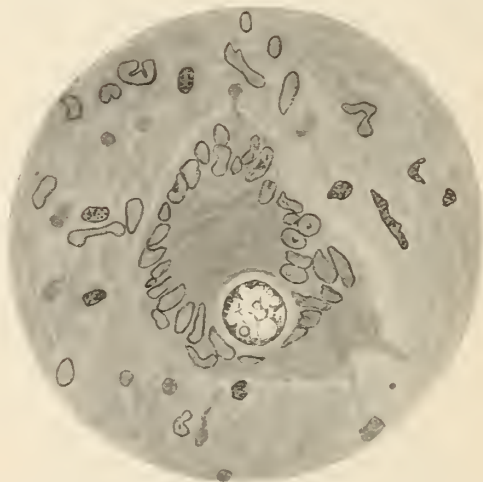


FIG. 6.—Sporulation within a giant cell. Well developed organisms are seen partly enclosed by the fragments of the ruptured capsule of the parent organism.  $\times 500$ .

ous forms and groups are seen which correspond to the sporulation forms described by Rixford and Gilchrist and by Ophüls. In these the number of spores varies, but for the most part is very large, 100 or more from a single parent organism. These sporulation forms are confined to the minute abscesses in the center of which they are usually found. The capsule is thin and flexible, and in section is shown wrinkled or undulating (Figs. 4, 5). A large group of recently liberated organisms is also shown in Fig. 7. Some of these groups are very large, evidently containing several hundred small organisms.

In this tissue, however, another phase of endogenous sporulation is also seen and the instances are more numerous than of that type

just described. In these forms the number of new formed parasites within the parent capsule does not exceed twenty, and the "spores" are of relatively larger size than those described above. The capsule here is always thick and well defined, is apparently brittle, and is not capable of wrinkling, but when it bursts from the pressure of the developing spores within, separates into several fragments, which retain their original form. This is shown in Figs. 3 and 6.

As they are seen in the unruptured capsule, or in one just beginning to rupture, most of the spores are not exactly spherical, but are apparently under pressure and accommodate their shape to their surround-

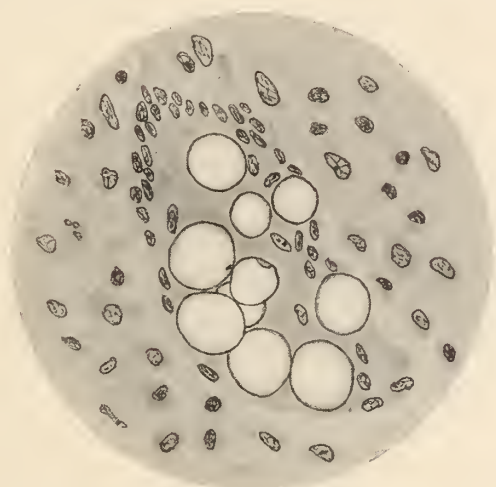


FIG. 7.—Giant cell containing a number of organisms without granular cytoplasm. These have apparently recently been liberated by sporulation of their parent organism.  $\times 500$ .

ings. These new formed organisms for the most part appear not to contain a well developed cytoplasm, but more like a thin capsule containing a fluid. This is shown in the recently liberated organisms contained within the giant cell in Fig. 7. However, some of them are distinctly granular and a few show the typical structure of the organisms as illustrated by the two fully formed organisms within the fragments of the parent capsule in Fig. 6.

It is to be noted that this form of sporulation is seen only in the typical giant cells and nodules, and never within the minute abscesses, while the sporulation forms previously described are always surrounded by a minute abscess.

There is an invariable yellowish brown pigment within the organisms which are sporulating with the rigid capsule just described.

No budding forms can be seen in any of our sections, reproduction taking place evidently only by sporulation.

#### HYALIN BODIES IN GRANULATION TISSUE.

One interesting feature of the tissues in these lesions deserves special attention. Scattered throughout the granulation tissue are great numbers of peculiar, variously sized, perfectly spherical bodies. Unstained, they appear as clear, glassy, highly refractive bodies.

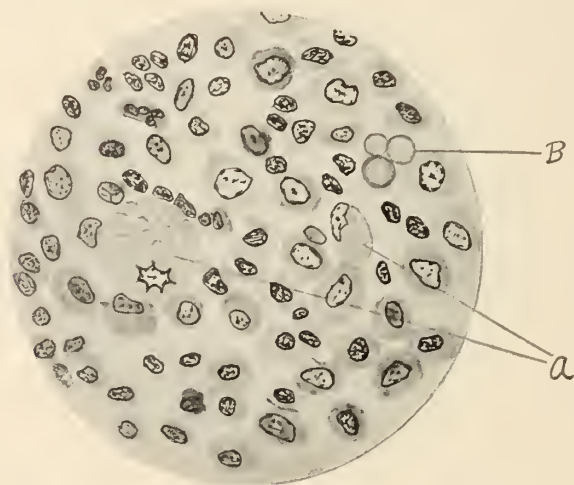


FIG. 8.—Groups of spherical hyalin bodies. A, small intracellular forms. B, large extracellular spherules.

Their affinity for stains seems to vary, the larger forms usually taking the different stains more deeply than the smaller. All, especially the larger ones, have a marked affinity for the fuchsin stain and retain a deep red color after decolorizing the sections with acids. Some retain Gram's stain, while others do not. With the ordinary hematoxylin and eosin stain they show a marked affinity for the red color. With polychrome methylene blue and certain other methylene blue preparations, the larger forms take a decided blue stain. Their size varies from minute forms up to 15-16  $\mu$  in diameter.

These hyalin bodies are contained within the cytoplasm of certain plasma cells for the most part, especially the smaller ones which are

seen in groups of 25 or 30 distending the cell wall and distorting the nucleus of the plasma cell in which they are present (Fig. 8). Where the larger ones are seen a much smaller number is present within a single cell, one cell containing one or more larger spheres. Many of the larger ones are free in the tissue spaces. These bodies are seen in greatest numbers in the areas of plasma and small round cells, and are especially numerous in the perivascular lymph-spaces. The hyalin bodies are not present in the nodules and giant cells.

Somewhat similar bodies are described by Ricketts in his monograph on *Blastomycetic Dermatitis*. Apparently he does not describe forms as large as those seen in this case. I have taken much interest in studying these, and it has occurred to me that they might possibly be related in some way to the organisms causing the lesion, instead of being, as Ricketts considers them, a hyalin degeneration of the cell protoplasm. However, I have come to no definite conclusion on this point. In sections from one of Dr. Litterer's cases of blastomycetic dermatitis which I have had opportunity to see, the tissues contained great numbers of these hyalin bodies, and this case was unusual in the fact that altho a well-defined case of typical blastomycetic dermatitis, it was exceedingly difficult to demonstrate the presence of the characteristic organisms of blastomycosis, thus leading to the suspicion that the hyalin bodies might be an unusual form of the parasite. Ricketts has described a somewhat similar occurrence in one of his cases (Case 1). I have also observed in a series of cases of chronic inflammatory tumors of the gums the invariable presence of these hyalin bodies. Other investigators have described apparently identical structures in the tissues of chronic inflammatory lesions and the general tendency is to regard them as originating from cytoplasmic degeneration or infiltration and not as parasitic organisms.

#### CONCLUSIONS.

The organism in the case reported evidently belongs to the class of sporulating fungi called *Oidium coccidioides*.

Altho the lesions microscopically resemble tuberculosis, the tubercle bacilli are not found.

In the few cases of involvement of the central nervous system reported, the coccidioidal disease apparently has a predilection for

the leptomeninx; while the majority of nervous lesions in systemic blastomycosis have been in deeper tissues of the brain.

It seems probable from the large number of cases of this apparently unusual disease discovered in the localities where certain investigators have been especially on the lookout for them, that if our attention were more frequently called to their possible occurrence they would be found to be more prevalent in other localities also.



# THE TOXIC AND ANTIGENIC PROPERTIES OF BACTERIUM WELCHII.\*

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## INTRODUCTION.

*Bacterium welchii* (Migula), or *Bacillus aerogenes capsulatus* (Welch), was discovered in 1891 by Professor Welch in the blood and tissues of a tuberculous person who had died of hemorrhage from the rupture of an aortic aneurism. The entire body was emphysematous and distended with gas. Subsequently this bacillus has been isolated in many pathological conditions, among which may be mentioned emphysematous gangrene and gaseous phlegmons of various tissues following injuries, surgical operations (herniotomy, nephrectomy, urethrotomy, appendectomy, amputations, etc.), salt infusions, hypodermic injections, and cholelithiasis.

It has also been found in emphysema of the fetus, puerperal eclampsia, physometra, emphysema of the uterine wall, puerperal gas sepsis, urethral stricture with cystitis, emphysema of the bladder, interstitial emphysema of the gastro-intestinal wall, pneumoperitonitis with or without perforation, pneumonia and pulmonary gangrene, and in meningitis.

Herter<sup>1</sup> has suggested that certain anemias, especially in children, may be associated with a chronic invasion of the intestinal tract by *B. welchii*.

In the light of the seeming increasing importance of *B. welchii*, it was deemed desirable to investigate the immunological relations of this bacterium.

As stated, *B. welchii* was discovered in 1891, the report of the discovery being published by Welch and Nuttall in 1892. Early in January, 1893, E. Fraenkel<sup>2</sup> described the same organism which he named *Bacillus phlegmones emphysematosae* because of its isolation from gaseous phlegmons. This work was followed by a more complete report of his studies in a monograph, "Ueber Gasphlegmonen." The same

\* Received for publication June 24, 1909.

<sup>1</sup> *Jour. Biol. Chem.*, 1906, 2, p. 1.

<sup>2</sup> *Centralbl. f. Bakt.*, 1893, 13, p. 13.

bacterium has been described by other European investigators who ignored the American work. Thus *B. enteritidis sporogenes* (Klein),<sup>1</sup> *B. perfringens* (Veillon and Zuber),<sup>2</sup> *Granulo-bacillus saccharobutyricus immobilis* (Schattenfroth and Grassberger),<sup>3</sup> *B. anaerobie cryptobutyricus* (Achalme),<sup>4</sup> *B. cadaveris butyricus* (Buday),<sup>5</sup> *Bacillus of Cesaris-Demel*,<sup>6</sup> and *B. emphysematis vaginae* (Lindenthal)<sup>7</sup> are undoubtedly *B. welchii*.

Welch in 1900<sup>8</sup> and E. Fraenkel in 1902<sup>9</sup> gave excellent and complete résumés of the literature. It is therefore necessary to refer only to a few observations made since their reports, and to discuss only certain points relative to the pathological changes produced by the organism. Of work dealing with certain phases of the immunity to and infection with *B. welchii*, that of Kamen, "Zur Aetiologie der Gasphegmon,"<sup>10</sup> dealing with toxins, agglutinins, etc., and of Passini, "Ueber Giftstoffe in den Kulturen des Gasphegmon-Bazillus,"<sup>11</sup> are noteworthy. Werner<sup>12</sup> discusses the agglutinins for the gas bacillus.

Falkner<sup>13</sup> deals with certain phases of the production of foamy organs, and Ghon and Sachs<sup>14</sup> with some of the gross pathological aspects of infections with *B. welchii*. The work of Herter<sup>15</sup> on the bacterial processes in the intestine in advanced anemias and the relation of *B. welchii* to them, and the studies of Rettger<sup>16</sup> on intestinal putrefaction and the relation of *B. welchii* to the various processes, are also worthy of consideration. Noguchi<sup>17</sup> has reported on "Sporulation in the Group of *Bacillus aerogenes capsulatus*" and Legros<sup>18</sup> on "Recherches histologiques sur les gangrenes gazeuses aiguës." A few other observations of interest will be mentioned under the section on pathology.

#### ISOLATION AND CHARACTERISTICS.

**Habitat.**—*B. welchii* is widely distributed, occurring most universally in the intestinal tract of man and animals, in sewage, soil, water, milk, etc. Dolby<sup>19</sup> found it in 69 of 250 blank cartridge wads, probably introduced in the ingredients used in manufacture.

**Method of Isolation.**—The method of isolation essentially suggested by Welch, with some slight modifications, is as follows: One gram of soil is shaken in sterile NaCl solution (0.85 per cent) and inoculated into sterile neutral litmus milk tubes

<sup>1</sup> *Centralbl. f. Bakt.*, 1895, 18, p. 737.

<sup>3</sup> *Munch. med. Wchnschr.*, 1900, 47, p. 1077.

<sup>2</sup> *Arch. de méd. expér.*, 1898, 10, p. 539.

<sup>4</sup> *Ann. de l'Inst. Pasteur*, 1897, 11, p. 845.

<sup>5</sup> *Centralbl. f. Bakt.*, 1898, 24, p. 369.

<sup>6</sup> *Giornale d. r. Accad. di Med. di Torino*, 1898, 61, p. 1256; 62, p. 190, cited by Welch.

<sup>7</sup> *Wien. klin. Wchnschr.*, 1897, 10, p. 3.

<sup>8</sup> *Bull. Johns Hopkins Hosp.*, 1900, 11, p. 185.

<sup>9</sup> *Erg. der Path.*, 1902, 8, p. 403.

<sup>10</sup> *Centralbl. f. Bakt.*, 1904, 35, pp. 554, 686.

<sup>11</sup> *Wien. klin. Wchnschr.*, 1905, 18, p. 921.

<sup>12</sup> *Arch. f. Hyg.*, 1904, 50, p. 274; 1905, 53, p. 128.

<sup>13</sup> *Beiträge zur Frage der Schaumorgane*, Zürich, 1905.

<sup>14</sup> *Centralbl. f. Bakt.*, 1903, 33, p. 657.

<sup>17</sup> *Trans. N. Y. Path. Soc.*, 1907-1908, 7, p. 196.

<sup>15</sup> *Jour. Biol. Chem.*, 1906, 2, p. 1.

<sup>18</sup> *Arch. de méd. expér.*, 1903, 15, p. 1.

<sup>16</sup> *Ibid.*, 1908, 4, p. 45.

<sup>19</sup> *Jour. Amer. Med. Assoc.*, 1905, 44, p. 466.

which are covered with 25 mm. of neutral paraffin oil for the purpose of securing anaerobiosis, and then incubated for 24 hours at 37° C. At the end of this time the milk in the tube is coagulated and shows acid and gas (see milk-infra). A sub-culture is made in a second litmus milk tube under oil and incubated for 12 hours in order to prevent the possible overgrowth of other bacteria. At the end of this time the milk usually shows coagulation, acid, and gas production as in the first instance ("stormy fermentation"); 0.5 c.c. of the whey in the sub-culture is then injected into the posterior auricular vein of a rabbit. In three or four minutes the animal is killed by a blow on the head and the body incubated at 37° C. for 8 to 10 hours, at the end of which time the abdomen is markedly distended with gas. When ignited this gas explodes and burns with a hydrogen flame. The thorax of the animal is carefully opened and cultures made from the heart blood in dextrose broth covered by neutral paraffin oil. In from 8 to 24 hours the culture tubes show a marked cloudiness, abundant gas production, and, in most instances, an odor of butyric acid. With this technic a pure culture of *B. welchii* is usually secured.

**Characteristics of *B. welchii*.**—This bacterium is an obligate anaerobe. Typical cultures show the following characteristics. It is a non-motile, rod-shaped bacterium, from 3 to 6  $\mu$  long. I have noted shorter forms (2–3  $\mu$ ). Occasionally it may be quite broad and plump but usually it is from 5–8  $\mu$  in width. It frequently grows in chains but is often single and in pairs. In the fluids and organs of the body capsules are usually produced, but seldom on artificial media. Spores are often produced, but in some strains spore production does not seem to occur. The spores usually appear in the center of the rod, altho occasionally at the end, and markedly increase the diameter of the cells at these points. The spores stain with comparative ease by ordinary methods.

The asporogenous type of *B. welchii* may be transformed into the sporogenous type in various ways (Passini).<sup>1</sup> Grassberger inoculated sterile beef muscle with a dextrose agar culture incubated in a Buchner tube. Egg-white with broth may be used. Sporulation once started may be kept up by continuous transference on dextrose agar. Often the presence of alkalies will induce spore formation. Spore-bearing cultures on injection into susceptible animals often become asporogenous.

*B. welchii* stains by all the anilin dyes and by Gram's method.

Agar and gelatin plates (plain) show round, flat, greyish-white, smooth and semi-translucent colonies on the surface (1.5–4 mm.). Microscopically these colonies usually show a definite center or nucleus surrounded by a granular growth. The colonies are often "woolly" in appearance, similar to those of *B. tetani* (Muir and Ritchie). Deep colonies are round and white. Slope agar shows yellowish-white and extremely thin growth. Glucose agar and glucose gelatin (deep punctures) show abundant gas formation which splits the media into many parts.

Gelatin is slowly liquefied by some strains, the digested material showing proteoses and peptones.

Broth is rapidly clouded and a heavy precipitate and growth collects in the bottom. The medium becomes acid to litmus and occasionally shows gas production. Dextrose broth gives marked gas.

On potato growth usually fails. Occasionally some growth and a small amount of gas production are noted.

<sup>1</sup> *Wien. klin. Wchnschr.*, 1905, 18, p. 921.

Blood serum shows a greyish-white growth with a slight amount of digestion along the line of inoculation.

Milk, in 24 to 48 hours, shows casein, acid, and gas. The curd is riddled with gas bubbles, the so-called "stormy fermentation," and a distinct butyric acid odor is present.

**Gas Production.**—Gas production results in dextrose, lactose, and saccharose media, but according to my observations not in media containing mannite. This observation is at variance with that of some observers. Gas production sometimes occurs in media containing only the muscle sugar of the beef. Limited gas production also takes place in media containing only proteins, as originally pointed out by Welch. I was able to demonstrate this property in a one per cent solution of pure egg-white in a six-tenths per cent normal sodium chloride. The pure egg-white used was prepared after the method of Hopkins and three times recrystallized.

Gas production is much more rapid with *B. welchii* than with *B. coli*.

The gas produced is mainly carbon dioxide and hydrogen. According to Dunham the gas has the following composition:

	Hydrogen	64.3%
	Carbon dioxide	27.8%
	Nitrogen, etc.	8.1%

Various other analyses have been made with slight variation.

**Acid Production.**—In cultures in dextrose broth the odor of butyric acid is quite marked and the cultures are markedly acid. Lactic acid is also shown to be present by Uffelmann's reagent. Rodella<sup>2</sup> states that all anaerobic bacteria develop organic acids. Expressed in terms of propionic acid and calculating from the weight of barium salts, this writer found that *B. welchii* produced 0.257 grams acid for 100 per cent culture media (0.104 acetic, 0.153 butyric).

**Saccharo-butyric Fermentation and Putrefaction.**—*B. welchii* is principally a zymogenic bacterium but possesses in addition saprogenic properties which are reduced or absent in some strains. Methylmercaptan and aromatic oxy-acids are usually produced.

As mentioned, the sugars are fermented with the possible exception of mannite; consequently gas and some organic acids, such as butyric, acetic, and lactic, are produced. Butyric acid is the principal fatty acid present and its odor is noticeable in fluid and cultures containing the organism. Rettger<sup>3</sup> substantiates Bienstock's<sup>4</sup> statement that obligate anaerobes are responsible for the putrefactive changes in the native proteins. He also found that while the bulk of the proteins is decreased the process never assumes the character of true putrefaction, and that gas can be produced in the absence of carbohydrates. Welch made this observation and so have I. Fermentation and putrefaction of this kind go on in the proteins of the body with a post-mortem invasion of *B. welchii*. Fermentation is undoubtedly due to the presence of carbohydrate radicles in the proteins.

**Thermal Death Point.**—According to my observations, which are in agreement with those of other writers, the thermal death point for the vegetative forms of *B. welchii*

<sup>2</sup> Bull. Johns Hopkins Hosp., 1897, 8, p. 68.

<sup>3</sup> Ann. de l'Inst. Pasteur, 1905, 19, p. 804; Centralbl. f. Bakt., 1903, 33, p. 135.

<sup>4</sup> Jour. Biol. Chem., 1908, 4, p. 45.

<sup>5</sup> Arch. f. Hyg., 1899, 36, p. 335; *ibid.*, 1901, 39, p. 390

is 56° C. for 10 minutes. The spore-bearing cultures were found to be killed at an average of 100° C. for 15 minutes.

**Pathogenic Power and Lesions Produced by *B. welchii*.**—Among the lower animals infections by *B. welchii* are rare under natural conditions. Gaseous phlegmons have been observed by Harris<sup>1</sup> in rabbits and dogs following injuries. It has been stated by many writers that rabbits and mice are practically immune to artificial inoculation and that guinea-pigs and pigeons are susceptible. I have noted in my experiments that some rabbits possess a certain degree of immunity even when large numbers of bacteria are injected directly into the circulation, but that guinea-pigs are readily susceptible. Rabbits, however, are not immune to the extent most writers infer. I have repeatedly observed rabbits to succumb to intravenous inoculation, no point of necrosis or disease being found post mortem, as claimed by some to be necessary for infection.<sup>2</sup> Rabbits do not succumb to subcutaneous inoculation. As before mentioned, dead rabbits furnish an excellent medium for the growth of *B. welchii*, but this may be due to a loss of the bactericidal power of the blood.

The pathogenic properties of different strains of *B. welchii* vary for guinea-pigs when injected subcutaneously. Some strains are harmless, some produce death, and some subcutaneous hemorrhagic emphysema, edema, and necrosis.

One of the important questions often raised in connection with *B. welchii* concerns its invasion of the blood and tissues of the body. In a large percentage of general infections with this organism, the gas production and other changes are noted only after death. The tissues reveal a peculiar protein digestive process, are crepitant and emphysematous, filled with inflammable gas. The organs, especially the liver, may be riddled with gas cysts.

*B. welchii* has been observed in the blood one hour after death, but, as Welch states, this is sufficient time for the bacteria which have gained entrance into the tissues to multiply and produce gas. If we bear in mind the fact that *B. welchii* is a normal inhabitant of the intestinal tract of man and some animals, and may produce local lesions in this tract (Howard), it is easy to see how the tissues may be rapidly invaded from this source. It may be stated in this connection that in rabbits injected intravenously with milk-whey (0.25 c.c.) containing *B. welchii* and killed in three minutes and incubated at 37° C. for one hour, I have observed in the liver a loss of nuclear staining power around the interlobular vessels and in some instances minute gas cysts. It seems probable that *B. welchii* never produces gas in the blood in the living body and consequently is not a factor in the production of gaseous emboli as has been supposed.

At all events there are no well-authenticated cases on record of a generalized digestion of tissues or intravascular gas production ante mortem. Welch mentions several cases in the older literature but these lack substantiation. If there is present anywhere in the body, but usually on the exposed surfaces, any necrotic tissue *B. welchii* may grow with rapidity and produce the characteristic saccharo-butyric fermentation with slight amounts of gas production. However, in these cases the tissue is for all practical purposes dead and separated from the rest of the body. It seems, therefore, that the body fluids under ordinary conditions are antibacterial or do not furnish the nutrient requirements which are supplied post mortem. This condition is rather

<sup>1</sup> Cited by Welch, *Bull. Johns Hopkins Hosp.*, 1900, 11, p. 185.

<sup>2</sup> Welch states that rabbits withstand large inoculations unless there exists necrotic or damaged tissue somewhere in the body which offers no resistance to infections.



difficult to conceive, altho it has been shown, for example, that antibacterial properties of sera in snake venom poisoning entirely disappear post mortem.<sup>1</sup> Some observers claim to have isolated *B. welchii* from the blood during life; in such cases the antibacterial influences may have been sufficient to destroy the aerogenic and to attenuate the pathogenic properties of the organism. No gas production was observed in these cases and an etiological connection between the organisms and the pathological lesions could not be established.

Since Welch's<sup>2</sup> article in 1900 on the subject of "The Morbid Conditions Produced by *Bacillus Aerogenes Capsulatus*" the organism has been reported in many of the conditions noted by him. Among the more interesting may be mentioned those reported by Chosky,<sup>3</sup> Uffenheimer,<sup>4</sup> Holmsen,<sup>5</sup> Mooers,<sup>6</sup> Blynie,<sup>7</sup> Eagleton,<sup>8</sup> Puneo,<sup>9</sup> Sappington,<sup>10</sup> Dudgeon,<sup>11</sup> Cotton and Blake,<sup>12</sup> Coley,<sup>13</sup> Whitacre,<sup>14</sup> Loving,<sup>15</sup> and Schultz.<sup>16</sup>

One of the most interesting and suggestive articles dealing with *B. welchii* is by Herter.<sup>7</sup>

Herter raises the question as to the relation of primary pernicious anemia and some of the intestinal bacterial processes. Marked derangements of digestion and increase of *B. welchii* in the intestines are present in this disease and Herter suggests that the resulting saccharo-butyric putrefaction may give rise to toxic products that cause anemia. Herter finds that as the number of *B. welchii* increases in the intestine the number of *B. coli* diminishes and that there is also a simultaneous decrease in the erythrocytes. *B. coli* then would seem to hold *B. welchii* in check. Herter further holds that all gram-positive anaerobes in the intestine are *B. welchii* or sub-varieties.

#### THE TOXIC SUBSTANCES PRODUCED BY *B. WELCHII*.

In an investigation of immunity to *B. welchii* it is, of course, essential to have a clear understanding if possible of the nature of the toxic substances which this organism produces under various conditions.

In order to ascertain whether toxic substances are produced by *B. welchii* the organism was cultivated from garden earth on the various media under strict or partial anaerobic conditions. The following media were used: agar, gelatin, plain broth, broth with peptone 1 per cent, dextrose broth (1 per cent peptone, 1 per cent dextrose), milk, and blood serum.

<sup>1</sup> Ewing, *Lancet*, 1804, 1, p. 1236.

<sup>2</sup> *Bull. Johns Hopkins Hosp.*, 1900, 11, p. 185.

<sup>3</sup> *Lancet*, 1901, 2, p. 1572.

<sup>4</sup> *Limousen, Méd.*, 1903, 27, p. 27.

<sup>5</sup> *Jour. Med. Soc. New Jersey*, 1904-1905, 1, p. 97.

<sup>6</sup> *Ibid.*, 1904-1905, 1, p. 274.

<sup>7</sup> *N. Y. Med. Jour.*, 1904, 79, p. 641.

<sup>8</sup> *Trans. Path. Soc.*, London, 1905, 56, p. 42.

<sup>9</sup> *Boston Med. and Surg. Jour.*, 1906, 155, p. 646.

<sup>10</sup> *Mobile Med. and Surg. Jour.*, 1907, 10, p. 304.

<sup>11</sup> *Beil. z. Path. Anat.*, 1902, 31, p. 383.

<sup>12</sup> *N. Mag. f. Lægeu.*, 1903, No. 5.

<sup>13</sup> *Boston Med. and Surg. Jour.*, 1903, 143, p. 329.

<sup>14</sup> *Lancet Clinic*, 1907, 58, p. 118.

<sup>15</sup> *Interstate Med. Jour.*, 1907, 14, p. 686.

<sup>16</sup> *Virch. Arch.*, 1908, 193, p. 419.

<sup>17</sup> *Jour. Biol. Chem.*, 1906, 2, p. 1.

It was found that dextrose broth undoubtedly is the best medium, at least in giving the maximum acidity, and in my experiments dextrose broth was used unless otherwise stated.

Intravenous injection of 0.3-0.5 c.c. of a 24-hour broth culture of *B. welchii* in 8 to 12 hours in rabbits (which according to some writers are immune) gave rise to distinct evidences of intoxication in the majority of animals. In 25 per cent of the rabbits tested with the above amounts, death, preceded by a marked dyspnea, stupor, and later by convulsions in some cases, resulted in from 10 to 12 hours. In a few rabbits death did not result for 48 to 96 hours after injection. The animals dying in the period of intoxication usually have in the beginning a temperature of about 104° F., followed by a fall to about 96° F. and death. Filtrates of cultures (Berkefeld, Pasteur-Chamberland) produced the same intoxication in rabbits but it required about 2-5 c.c. intravenously, to cause death.

It may be stated here that I have observed a great variation in the resistance of various rabbits to *B. welchii*. Two animals of same weight, sex, and litter may react differently. Intraperitoneal injections of cultures and filtrates in rabbits of different ages were not fatal in 90 per cent. Subcutaneous injection of 0.5 c.c. produced no effects. However, when injected subcutaneously with larger doses of 24-hour cultures (2-5 c.c.) gaseous phlegmons resulted in a few instances. With cultures three or four days old the toxic effects seemed to be more pronounced.

In guinea-pigs the toxic effects of cultures and filtrates were decidedly more pronounced than in rabbits, the intracardiac injection of 0.25 c.c. usually causing death in from six to eight hours. The corresponding amounts intraperitoneally usually produced similar results. Subcutaneous injection of cultures gave rise in a large percentage of cases to gaseous and gangrenous abscesses, and to death in a short period.

All experiments were carefully controlled by the injection of uninoculated culture media.

**The Action of Heat on the Toxic Substances.**—Of the heated filtrate of a 48-hour dextrose broth culture 0.5 c.c. was injected intraperitoneally and intracardiacly in guinea-pigs of the same age and weight (Table 1). An equivalent amount of dextrose broth alone gave no symptoms.

TABLE 1.  
TOXICITY OF HEATED CULTURES.

Temperature	Time	Result	
50° C.	20 min.	Toxic	
50°	60 "	"	
60°	" "	"	
70°	" "	"	Slightly reduced after 2 hrs.
90°	" "	"	Reduced
100°	" "	"	Greatly reduced
100°	120 "	Slightly toxic	

This experiment, repeated with similar results, admits of but one interpretation. The toxic substance or substances in question do not seem to be true bacterial toxins because they are not completely destroyed by heating at 80° C. or above for 60 minutes. It concerns a substance which resists 90° C.-100° C., at which temperature it is rendered partially inactive. A temperature of 100° C. for two hours destroyed the toxicity nearly completely.

On heating with a reflux condenser a definite amount of a 48-hour culture and duplicating the foregoing experiment a slight reduction only in the amount of toxicity occurred after two hours, showing quite conclusively that the toxic substances had been volatilized to a large extent in the preceding experiments.

**The Acids in the Cultures.**—Now cultures in milk or in sugar-broth solutions in a short period reveal a distinct odor of butyric acid. It was therefore deemed advisable to test the cultures as to acidity (Table 2).

TABLE 2.  
DETERMINATION OF ACIDITY OF CULTURES IN DEXTROSE BROTH.

Days	Titration A	Titration B	Titration C	Average	Media	Acid
1.....	.024%	.026%	.025%	.025%	.008%	.017%
2.....	.037	.037	.038	.037	"	.024
3.....	.038	.038	.038	.038	"	.030
4.....	.038	.038	.037	.037	"	.029
5.....	.035	.034	.035	.034	"	.026
12.....	.022	.021	.025	.022	"	.024
25.....	.015	.015	.020	.016	"	.008
30.....	.015	.017	.016	.016	"	.008
60.....	.015	.016	.015	.015	"	.007

Titration made with  $\frac{N}{10}$  NaOH, phenolphthalein indicator. Incubation 37° C.

The titrations were made with three different cultures, A, B, C, in the same medium. A few cultures not included in this series showed no decrease in the amount of acid produced in 60 days (0.025 per cent was noted in one case). The normal for the culture medium remained 0.008 per cent in the whole series. From the foregoing table it is seen that the amount of acid present increases up to a

certain point and then diminishes but at 60 days the acid has not been completely neutralized or decomposed. The decrease in the acidity may be owing to the formation of ammonia compounds, or the decomposition of the acid by the bacterial secretions. It is known that butyric acid organisms often produce substances which will decompose the butyric acid formed.

The next step taken was the determination of the acids in the cultures. A cursory examination by the usual qualitative methods showed butyric acid to be the principal acid present with acetic and lactic acid in smaller amounts. In all probability other organic acids also occur in smaller amounts. It is impossible to tell the exact percentage of acidity due to butyric acid.

**The Toxic Action of Butyric Acid Compared with Toxic Action of Culture Filtrates.**—The question as to what part of the toxic action of *B. welchii* is due to these organic acids naturally arises.

Normal butyric acid (Kahlbaum) was used in the following experiments in which guinea-pigs were injected. The exact nature of the toxic action of butyric acid is discussed subsequently.

TABLE 3.  
THE TOXIC ACTION OF NORMAL BUTYRIC ACID FULL STRENGTH (100) ON INTRACARDIAC INJECTION IN GUINEA-PIG.

Amount of Acid	Per cent Acid	Result
0.25 c.c.	12.3	Death 1 min.; convulsions
0.15 "	"	" 2½ " "
0.1 "	"	" 2½ " "
0.05 "	"	" 15 " "
0.025 "	"	Severe symptoms—convulsions slight

Titrations made with N/1 NaOH, phenolphthalein indicator.

Intraperitoneal injections of 0.25 c.c. of full-strength butyric acid caused marked peritoneal irritation followed by recovery and subcutaneous injection of the same amount caused a necrotic inflammation also followed by recovery.

TABLE 4.  
THE TOXIC ACTION OF BUTYRIC ACID 1 : 500 ON INTRACARDIAC INJECTION IN GUINEA-PIG.

Amount of Acid	Per cent Acid	Result
0.25 c.c.	.021	Respiratory spasms, dyspnea 3 min., may recover after several hours.
0.15 "	"	Respiratory spasms, dyspnea, 3 min. 30 sec., may recover after several hours.
0.1 "	"	Respiratory spasms, dyspnea 4 min., may recover after several hours.
0.05 "	"	} No symptoms except very slight irritation.
0.025 "	"	

Intraperitoneal and subcutaneous injections of 0.25 c.c., 1:500 butyric acid gave no effects.

Normal butyric acid 1:500 shows an acidity of about 0.021 per cent, i. e., approximately about the same amount of acid as is produced in a 24-hour culture of *B. welchii* in dextrose broth.

TABLE 5.  
THE TOXICITY OF BUTYRIC ACID FULL STRENGTH ON INTRAVENOUS INJECTION IN RABBITS.

Amount of Acid	Per cent Acid	Results	
0.25 c.c.	12.3	Death 3 hours.	Convulsions.
0.15 "	"	" 12 "	"
0.1 "	"	" 15 "	" mild.
0.05 "	"	Severe symptoms, often eventual death.	
0.025 "	"	Symptoms less severe, occasional death.	

TABLE 6.  
THE TOXIC ACTION OF BUTYRIC ACID 1:500 ON INTRAVENOUS INJECTION IN RABBITS.

Amount of Acid	Per cent Acid	Results
0.25 c.c.	0.021	Severe symptoms, occasionally die. Respiratory paralysis, dyspnea.
0.15 "	"	Severe symptoms, usually recover.
0.10 "	"	Symptoms less severe, tremors.
0.05 "	"	Slight fever.
0.025 "	"	No symptoms.

Some irritation on intraperitoneal and subcutaneous injection of 0.25 c.c.; no effect with injections of 1:500.

A comparison of these two series of experiments shows the difference in the susceptibility of guinea-pigs and rabbits to normal butyric acid. The same condition occurs on injection of cultures and of filtrates of *B. welchii*. In rabbits 0.3-0.5 c.c. of cultures (24-48 hrs.) produce intoxication on intravenous injections (acidity 0.025-0.037 per cent) and normal butyric acid 1:500 intravenously in doses of 0.25-1.00 c.c. (acidity 0.021 per cent) produces similar results.

Guinea-pigs and occasionally rabbits on subcutaneous injection of normal butyric acid full strength often develop deep phlegmons which break and suppurate. The tissue in the region of the acid becomes rapidly necrotic. In the beginning sterile pus is present, but later mixed infections occur.

After carefully neutralizing the filtrate of 48-hour cultures of *B. welchii* with 0.1 per cent sodium bicarbonate and also by 0.05 per cent sodium hydroxide and injecting in a series as given above, uniformly negative results were obtained.



**Inoculation with Washed Bacteria.**—Twenty-four hour cultures grown in dextrose broth and centrifugated, washed three times in 0.85 per cent NaCl solution, were injected in doses of 0.25–0.5 c.c. in 0.85 per cent NaCl, intravenously and intraperitoneally in guinea-pigs and rabbits. In rabbits no symptoms resulted; a few guinea-pigs inoculated by intracardiac method showed signs of slight intoxication.

**Autolytic Extracts of *B. welchii*.**—Broth cultures were grown under paraffin oil, precipitated by centrifugalization, and subsequently washed three times with 0.85 per cent NaCl solution. The precipitate was then placed in a sterile mechanical ball mortar and ground for one and two hours until the bacteria were disintegrated. The material was now allowed to digest from four to six hours at 37° C. and tested as follows:

A thick emulsion of the ground bacteria was made in 0.85 per cent NaCl solution and varying amounts (0.1–1.00 c.c.) injected in rabbits and guinea-pigs both by intraperitoneal and intravenous methods. The rabbits showed no symptoms and several guinea-pigs showed only slight evidences of intoxication. No distinct toxin could be isolated by filtration. On digesting the material for several days and injecting guinea-pigs and rabbits during this period, no toxic substance could be demonstrated.

**Previous Observations on the Toxic Substances.**—Passini<sup>1</sup> describes some of his observations on the toxic substances produced by *B. welchii* (*B. phlegmones emphysematosae* Fraenkel). He states that this organism produces two distinct toxic substances: one which causes rapid death in animals by disturbances of respiratory and circulatory centers and another which causes vomiting, later diarrhea and death in from 10 to 12 hours. These toxic substances are produced in 14 to 30 days at 37° C. Passini states that 0.5 c.c.–1.5 c.c. of Pukall-filtrate causes death in a rabbit weighing one kilo in 30 to 60 seconds when injected intravenously. These animals show two types of intoxication. Some have motor disturbances during which the animal races across the room colliding with many obstacles and later develops convulsions and dies. Others after 10 to 20 seconds become paralyzed in the head and limbs and later death occurs. Mechanical irritation is attended by some incoordinate response; non-fatal doses produce intoxication. Subcutaneous injection of 3 c.c. of a filtrate into 250–300 gram guinea-pigs is followed by similar symptoms. Passini notes a variation in the centers attacked by toxins derived from different strains. For example, one culture derived from the feces of a patient with pernicious anemia produced a toxic substance which acted primarily upon the respiratory center and another procured from Kral's laboratory acted primarily on the circulatory and nervous systems.

<sup>1</sup> *Wien. klin. Wchnschr.*, 1905, 18, p. 921.

This irregularity suggests different organisms. Furthermore, Passini's medium was prepared by digesting beef muscle with trypsin. He does not consider the possibility of the proteoses and peptones, etc., produced in this process being toxic in themselves. Neither does he consider the acid produced a factor. He states, however, that if one per cent dextrose is used that the "acid end product" is toxic. The same writer states that some media on standing three or four weeks at 37° C. become distinctly alkaline and that on subcutaneous injection of 3 c.c., hemorrhagic edema is produced. He also notes that 60° C. for one hour or 100° C. for 15 minutes does not impair the toxic action of the filtrate to any great degree. He isolated no distinct toxin and does not elucidate any of the problems of immunity.

Kamen found that filtrates of cultures from a surgical phlegmon were faintly hemolytic and leukolytic for the blood cells of man, dogs, guinea-pigs, and rabbits. He could isolate no distinct toxin or lytic agent.

Herter<sup>2</sup> states that *B. welchii* produces a moderately toxic (hemolytic) substance. Heating at 70° C. for one hour with exhaustion reduced the toxic substance in its activity and 100° C. does not destroy it according to this author. No distinct toxin could be isolated even after careful neutralization of the acid. Herter's work will be referred to again in the discussion of the specific action of the toxic substances.

E. Fraenkel<sup>3</sup> and Hitchman and Lindenthal<sup>4</sup> among others failed to isolate any toxin from *B. welchii*.

#### HEMOLYSINS PRODUCED BY *B. WELCHII*.

It has been stated by some investigators that *B. welchii* produces hemolytic substances. The nature of these substances has never

TABLE 7.

THE ACTION OF FILTRATE OF 24-HOUR CULTURE (0.025% ACID) ON NORMAL RABBIT CORPUSCLES.

AMOUNT OF FILTRATE	HEMOLYSIS		
	Filtrate 1	Filtrate 2	Filtrate 3
0.5 C.C. ....	complete	complete	complete
0.25 " .....	al. complete	al. complete	complete
0.1 " .....	marked	marked	al. complete
0.05 " .....	medium	medium	marked
0.025 " .....	o	slight	slight
0.01 " .....	o	trace	trace
0.005 " .....	o	o	trace (?)
0.0025 " .....	o	o	o
Culture medium 0.5 C.C. ....	o	o	o

Filtrates Nos. 1, 2, and 3 were from three strains of *B. welchii*.

been satisfactorily explained. The following experiments were made in the hope of gaining accurate information on these so-called hemolysins.

The erythrocytes used were in 5 per cent suspension and washed

<sup>1</sup> *Centralbl. f. Bakt.*, 1904, 35, p. 686.

<sup>2</sup> *Jour. Biol. Chem.*, 1906, 2, p. 1.

<sup>3</sup> *Ergebnisse der Path.*, 1902, 8, p. 403.

<sup>4</sup> *Sitzung d. k. Akad. d. Wiss. Wien*, 1901, 110, Sep.

three times with 20 volumes of 0.85 per cent NaCl solution. The hemolytic mixtures were incubated for one hour at 37° C., shaken, and again incubated one hour, when the tubes were shaken again and placed in the ice chest for 12 hours. Hemolysis usually occurred in from 30 to 60 minutes. Each tube contained 1 c.c. of a 5 per cent suspension of rabbit corpuscles, the indicated amount of filtrate, and enough NaCl solution to make the total quantity 2 c.c.

Similar experiments with whole cultures gave the same results and the corpuscles of injected rabbits were affected in the same way as normal corpuscles. Tubes containing filtrate (0.25-0.5 c.c.) often show a marked precipitate of proteins.

It is important to note that in these experiments really typical hemolysis did not occur altho all the erythrocytes were disintegrated. In tubes indicated as "complete, almost complete," etc., the color was not the typical reddish-cherry color but a reddish- or yellowish-brown and in some cases a brownish-violet color. Tubes of this kind showed on spectroscopic analysis, methemoglobin. I have determined that this appearance is probably due to the organic acids produced by the bacteria in the culture before filtration. Mixtures in the hemolyzed tubes are distinctly acid to litmus. The same color is noted when pure butyric acid (Kahlbaum) is mixed with 0.5 per cent rabbit or guinea-pig erythrocytes.

Having observed that guinea-pigs are much more susceptible to infections with *B. welchii* than rabbits, I next tested in the same way the effect of 24- and 72-hour cultures on the erythrocytes of the guinea-pig.

TABLE 8.  
THE ACTION OF FILTRATE OF 24-HOUR CULTURES ON NORMAL GUINEA-PIG CORPUSCLES.

AMOUNT OF FILTRATE		HEMOLYSIS		
		Filtrate 1	Filtrate 2	Filtrate 3
0.5	c.c.	complete	complete	complete
0.25	"	"	"	al. complete
0.1	"	al. complete	al. complete	"
0.05	"	"	"	marked
0.025	"	marked	marked	slight
0.01	"	medium	slight	trace
0.005	"	trace	trace	o
0.0025	"	o	o	o
Culture medium 0.5 c.c.		o	o	o

Cultures and filtrates (48-72 hours old, 0.037-0.038 per cent acid) gave practically the same results. Methemoglobin was produced as in case of rabbit corpuscles.

Cultures and filtrates were tested on 5 per cent suspensions of the erythrocytes of the pigeon, sheep, goat, and monkey also, and a typical hemolysis was produced in each case. In the case of the goat blood there was a marked protein precipitate in the tubes containing the largest amount of culture or filtrate. Its nature was not investigated.

**The Action of Normal Butyric Acid 1:500 (0.021 per cent) on Normal Rabbit Corpuscles.**—Since it has been shown that the butyric and closely allied acids which are produced by *B. welchii* in culture under certain conditions are toxic for rabbits and guinea-pigs, the action of this acid upon the erythrocytes of these species was studied.

TABLE 9.  
THE LYTIC ACTION OF BUTYRIC ACID 1:500 ON RABBIT CORPUSCLES.

Amount of Acid	Lysis
1.00 c.c.	complete
0.5    "	"
0.25   "	al. complete.
0.15   "	"   "
0.1    "	slight
0.05   "	0

Each tube contained 1 c.c. 5 per cent suspension of corpuscles, the amount of acid 1:500 indicated, and enough NaCl solution to make 2 c.c. The yellowish-brown methemoglobin color was prominent, since pure acid was used.

**Neutralization of Filtrates of Cultures.**—The filtrates of cultures and the cultures themselves being hemolytic and methemoglobin being produced by butyric and allied organic acids, the next step was carefully to neutralize the acid in the cultures and then test their lytic action on various erythrocytes.

For this purpose 0.1 per cent sodium bicarbonate in 0.85 per cent NaCl (non-hemolytic for guinea-pig and rabbit corpuscles) was used with phenolphthalein as an indicator. A solution of 0.05 per cent NaOH was also used for the purpose of neutralization, but it was not satisfactory as the hydroxyl ions (OH) are so strongly hemolytic that even with the most careful technic in neutralization enough excess of NaOH may result to cause lysis.

The neutralized filtrates of 24-72 hour cultures were found to have no lytic action on rabbit and guinea-pig erythrocytes in several series of tests. Sometimes, however, tubes containing 0.5 c.c. and 0.25 c.c. filtrate seemed to show the presence of an agent producing slight

typical hemolysis. On heating the neutralized filtrates for 30 minutes at 80° C. the lytic agent entirely disappeared. The possibility of the presence of a typical bacterial hemolysin naturally suggested itself, but since the thermolabile hemolytic agent was found in two series only the lysis may have been due to some unknown fault in technic, such as an excess of the neutralizing agent and liberation of carbon dioxide.

Jordan<sup>1</sup> has shown that a hemolytic thermostable precipitate is produced in broth as it becomes alkaline. There is a possibility that such substances may be produced in this case although none have been demonstrated.

Herter<sup>2</sup> states that *B. welchii* produces moderately active hemolytic substance or substances. He carefully neutralized 0.5 c.c. of filtrate and found that it induced hemolysis in rabbit and monkey (Rhesus) erythrocytes. He treated the filtrate by exhaustion and it reduced the hemolytic action slightly. Heating to 70° C. for an hour reduced it still further. He also states that boiling does not destroy the hemolytic agent. In order to ascertain whether hemolysis was due to volatile ammonia compounds, Herter rendered the filtrates of the cultures alkaline with NaHCO<sub>3</sub>, concentrated it at reduced pressure to drive off ammonia, then restored the volume with 0.85 per cent NaCl. He states that this procedure reduced the hemolytic action of the filtrate somewhat. Herter does not seem to regard the acids present as factors or to appreciate the fact that the NaHCO<sub>3</sub> used in neutralization is a distinctly hemolytic substance in solutions over 0.1–0.5 per cent. Herter's report does not give details as to method.

Kamen<sup>3</sup> found the filtrates of cultures hemolytic for the erythrocytes of man, dog, guinea-pigs, and rabbits. He does not refer to the acids produced and states that he could not isolate any definite toxic substance. The same is true of Passini,<sup>4</sup> altho he seems to think that the toxin is not similar to an ordinary bacterial toxin but more closely allied to the products of certain putrefactive yeasts such as described by Faust.<sup>5</sup>

<sup>1</sup> *Jour. Infect. Dis.*, 1905, 2, p. 511.

<sup>2</sup> *Jour. Biol. Chem.*, 1906, 2, p. 1.

<sup>3</sup> *Centralbl. f. Bakt.*, 1904, 35, p. 686.

<sup>4</sup> *Wien. klin. Wchnschr.*, 1905, 18, p. 921.

<sup>5</sup> Cited by Passini, *Wien. klin. Wchnschr.*, 1905, 18, p. 921.



**The Presence of Hemolysins in Rabbits Immunized with *B. welchii*.**—Rabbits were immunized with injections of 48-hour beef broth cultures, beginning with 0.1 c.c. and increasing the amounts to 1.00 c.c. The weight and temperature of the animals were used as guides to reinjection, which was made only when temperature and weight were normal.

The serum of rabbits two and three days after inoculation with 0.1 c.c. of 48-hour culture causes no lysis of normal rabbit corpuscles nor of washed corpuscles of the injected rabbits.

Ten days after the first inoculation of 0.1 c.c. and two days after the second injection of 0.015 c.c. of 48-hour culture, the sera of two rabbits in two series gave approximately the result in the one series given in Table 10.

TABLE 10.  
THE PRESENCE OF LYSIN IN INJECTED RABBIT SERUM FOR NORMAL RABBIT CORPUSCLES.

Amount of Serum		Hemolysis
0.5	c.c.	Slight
0.25	"	Trace
0.1	"	Faint trace
0.05	"	" "
0.025	"	0
0.1	"	0
0.005	"	0
0.0025	"	0
0.0	"	0

Each tube contained 1 c.c. of 5 per cent suspension of rabbit corpuscles, the amount of serum indicated, and enough NaCl solution to make 2 c.c. There was a slight brownish-yellow color of the fluid in the tube showing lysis.

A hemolytic agent having been demonstrated in this serum, it remained to determine its nature.

Twenty-four days after the first injection (0.1 c.c.), 12 days after the second injection (0.5 c.c.), and four days after the third injection (1.00 c.c.) of a 48-hour culture of *B. welchii* the serum of the same rabbit gave the results shown in Table 11.

TABLE 11.  
THE PRESENCE OF LYSIN IN INJECTED RABBIT SERUM FOR NORMAL RABBIT CORPUSCLES.

Serum		Hemolysis
0.5	c.c.	Marked
0.25	"	Medium
0.1	"	Slight
0.05	"	Trace
0.025	"	Faint trace
0.01	"	0
0.005	"	0
0.0025	"	0
0.0	"	0

Heating the serum from this rabbit at 56° C. for one hour did not destroy the hemolytic agent. Heating to 70° C. for three hours completely inactivated the serum but it could not be reactivated by means of normal guinea-pig or rabbit serum as complement.

The tubes exhibiting hemolysis in all these experiments often showed a brownish-yellow coloration like that when the filtrates of cultures were mixed with normal rabbit corpuscles.

In order to investigate further the question of a thermostable complement, I treated the sera according to the method of Ehrlich and Morgenroth: The serum was mixed with  $\frac{1}{10}$  part of N/1 hydrochloric acid and digested at 37° C. for 45 minutes. It was then carefully neutralized with 0.1 per cent sodium bicarbonate. A series with the same rabbit serum as used in Tables 10, 11 showed no hemolysis in any of the tubes. The serum could not be reactivated by normal rabbit and guinea-pig serum as complement. The experiment practically eliminates the possibility of a thermostable complement and the hemolysis must be ascribed to some chemical agent, possibly the salt of an acid produced by the bacteria. A logical explanation for the negative results in the above-mentioned experiment is found in the fact that hydrochloric acid frequently decomposes organic acids, and even providing this did not occur all the acid salts would be neutralized by the sodium bicarbonate.

As is well known, normal rabbit serum is usually slightly hemolytic for guinea-pig erythrocytes. The serum from a rabbit which on being tested had shown the peculiar brownish-yellow hemolysin was heated at 56° C. for 30 minutes, thus destroying the normal complement. However, this serum still remained hemolytic for guinea-pig erythrocytes, showing the atypical nature of the hemolytic agent.

Since it has been shown that the cultures produce acid sufficient to cause lysis of rabbit corpuscles (atypical) and since there is a possibility of the acid in the culture not being rapidly excreted subsequent to injection but combining with some inorganic base in the serum and thus accounting for the hemolytic substances in the sera of immunized rabbits, I made some experiments with butyric acid.

It was found that in rabbits which recovered from injections of 1:500 normal butyric acid there was present two days after inoculation in the sera an agent which would produce the peculiar brownish-

yellow lysis of normal rabbit erythrocytes. This action was confined to the erythrocytes of other rabbits and I was unable to detect any intravascular hemolysis in the injected rabbit. This substance which was undoubtedly a salt of the acid in the culture injected was not present after three days.

It was noted that in the rabbits which had received several doses of cultures the hemolytic agent was present for a somewhat longer time. In regard to the presence of leukocytotoxic substances in the serum of rabbits injected with broth cultures of *B. welchii* see p. 557.

**Leukocytolysis.**—In connection with certain experiments on the phagocytosis of *B. welchii*, it was observed that the bacillus exerted a marked leukotoxic effect.

When the filtrates of 24- and 48-hour cultures of *B. welchii* and the washed leukocytes of the rabbit were brought together in capillary pipettes for periods varying from 2 to 15 minutes at 37° C., it was noted that lysis of the cytoplasm of the leukocytes was complete, together with marked vacuolization and loss of staining of the nuclei depending upon the time of contact. Broth alone had no effect on the cytoplasm of the leukocytes but produced a very slight vacuolization of the nuclei in some cases. Leukocytotoxic substances have also been described by Kamen<sup>1</sup> but were not isolated. When 24- and 48-hour cultures were used instead of the filtrates the same changes were noted in the leukocytes and there was no phagocytosis.

Equal parts of serum, washed leukocytes, and bacterial filtrate were mixed in the dilated portion of a small glass pipette, the pipettes sealed and incubated at 37° C. for varying periods. During the period of incubation the pipettes were occasionally rotated. Smears were stained with carbol-thionin. The leukocytes were obtained either from aleuronat pleural exudates or from blood cream, and were thoroughly washed with 0.85 per cent NaCl solution. It was noted that proportionately there were more polymorphonuclear leukocytes affected by the lytic substances than mononuclear leukocytes. The nuclei showed the most pronounced affects, there being vacuolization even after three minutes' incubation.

The vacuolization of the nuclei of the leukocytes in leukocytotoxin tests, etc., has also been noted by Eisenberg<sup>2</sup> in the case of two other

<sup>1</sup> *Centralbl. f. Bakt.*, 1904, 35, p. 686.

<sup>2</sup> *Compt. rend. Soc. de Biol.*, 1907, 62, p. 491; *Ann. de l'Inst. Pasteur*, 1908, 22, p. 430.

anaerobes, namely, *B. edematis maligni* and *B. anthracis symptomatica*, and by Gheroghiewsky<sup>1</sup> in *Ps. pyocyanea*. Eisenberg claims to have demonstrated an antileukocidin in symptomatic anthrax. Eisenberg regards his leukotoxin as a thermolabile substance and he states that chemotaxis is negative to all pathogenic anaerobes.

THE ANTIOPSONIC ACTION OF FILTRATES OF CULTURES OF  
B. WELCHII.

Hektoen<sup>2</sup> has shown that lactic acid acts as an antiopsonic substance preventing opsonins from sensitizing bacteria. He demonstrated that in the dilutions used, lactic acid does not affect the leukocytes or bacteria. Cultures of *B. welchii* contain butyric acid and some lactic acid, and consequently I undertook to investigate the effect of these substances. It was noted that when 24-hour broth cultures were used there was extensive leukocytolysis and no phagocytosis, while with washed bacteria there was phagocytosis. In order to study the action of the acid substances on the opsonins, the following experiment was made, modifying slightly the experiments made by Hektoen on lactic acid.

1. 0.2 c.c. serum + 0.05 c.c. filtrate (+0.03) incubated at 37° C. one hour, then added washed bacteria 0.5 c.c. + washed rabbit leukocytes and incubated at 37° C. for 30 minutes. No phagocytosis, leukocytolysis marked.
2. 0.2 c.c. serum + 0.05 c.c. (+0.03) filtrate + washed bacteria 0.5 c.c., incubated at 37° C. one hour; rewashed bacteria, then added washed rabbit leukocytes and incubated at 37° C. for 30 minutes. No phagocytosis.  
Controls (a) 0.2 c.c. serum + 0.5 c.c. washed bacteria + washed rabbit leukocytes incubated at 37° C. one hour. Phagocytosis. Some vacuolization.  
(b) 0.2 c.c. serum + 0.05 c.c. culture medium (+.008) incubated at 37° C. one hour, then added washed bacteria 0.5 c.c. + washed rabbit leukocytes, and incubated at 37° C. 30 minutes. Phagocytosis slight.  
(c) 0.2 c.c. serum + 0.05 c.c. NaCl + washed bacteria (0.5 c.c.) + washed leukocytes, incubated at 37° C. one hour. Phagocytosis slight.
3. 0.2 c.c. serum + 0.05 c.c. (+0.021) normal butyric acid, incubated at 37° C. for one hour, then added washed bacteria 0.5 c.c. + washed leukocytes, and incubated at 37° C. No phagocytosis. Leukocytolysis marked.  
Controls (a) Washed bacteria (0.5 c.c.) + 0.02 c.c. (+0.021) normal butyric acid and incubated at 37° C. for 30 minutes. Rewashed bacteria + serum 0.2 c.c. incubated at 37° C. one hour + washed leukocytes and incubated at 37° C. 30 minutes. Phagocytosis.

<sup>1</sup> *Ann. de l'Inst. Pasteur*, 1896, 10, p. 580.

<sup>2</sup> *Jour. Amer. Med. Assoc.*, 1906, 46, p. 1411.

The above experiments show, (1) that the acids in the culture filtrates are capable of inactivating the opsonins and that the culture media or sodium chloride alone has no effect. (2) That normal butyric acid (.021 per cent) is antiopsonic. (3) That normal butyric acid and filtrates of cultures have very little or no effect upon the bacteria and their subsequent sensitization by opsonins in 30 minutes and that these substances are distinctly lytic for the leukocytes.

Hektoen is inclined to believe that antiopsonic action is really responsible for the seeming negative chemotaxis toward bacteria and my observations seem to corroborate this and in addition demonstrate that the acids produced in culture are also lytic for leukocytes and consequently chemotaxis may be markedly influenced for this reason also.

Guinea-pig leukocytes show no difference from rabbit leukocytes as far as leukocytolysis is concerned, altho these animals are much more susceptible to infection by *B. welchii*. Phagocytosis, however, is more active with rabbit leukocytes than with guinea-pig leukocytes.

#### THE ANTIGENIC PROPERTIES OF *B. WELCHII*.

**The Opsonins for *B. welchii* in Normal and Immune Rabbit Sera.**—When normal serum, washed leukocytes, and washed bacteria are brought together in capillary pipettes at 37° C. for 10 to 15 minutes, a few of the leukocytes show a slight amount of lysis with vacuolization of the nuclei. An average phagocytosis of three or four bacteria may occur. On incubation for three to five minutes, two to four bacteria are taken up with only slight vacuolization of the nuclei and no lysis. No bacteria were taken up when serum was absent but when bacteria and leukocytes alone were in contact at 37° C. for 15 minutes a slight amount of lysis occurred. The results obtained with guinea-pig leukocytes were somewhat lower. It would seem that three or four bacteria were the maximum number which the leukocytes are able to take up.

It was found that the washed bacteria should be derived from cultures not over 24 hours old as the acid produced gives rise to marked precipitation of the proteins of the media and this interferes materially with securing a good emulsion of the washed bacteria.



Washed bacteria from the whey of milk cultures have been used with some success. Quite frequently, however, vacuolization of the nuclei occurs.

The rabbit used in one experiment (Table 12) had received three injections extending over a period of 20 days of 0.1 c.c., 0.5 c.c., and 0.5 c.c. of a 48-hour culture, the last one on the day before the serum was drawn.

TABLE 12.  
LEUKOCYTOTOXIC ACTION OF SERUM OF IMMUNIZED RABBIT.

Incubation	Leukolysis	Phagocytosis
15 min.	Marked	0
10 "	"	0.2
5 "	Vacuolization	0.5
3 "	"	0.8
3 "	" slight	0.7
15 " No serum, NaCl Sol.	0	

Table 12 demonstrates that there was some substance in the serum of the immunized rabbit in addition to that contained in the bacteria, which was leukocytotoxic, and consequently phagocytosis was not active, only a few leukocytes containing bacteria. The bacteria used were from the same strain used in previous experiments and were grown in exactly the same culture media. Since we know that the salts of butyric acid are not readily eliminated from the body of the rabbit, and as the rabbit had received the last injection only one day previous to the experiment, the leukocytolysis is accounted for easily. Heated serum (60° C. 30 min.) caused vacuolization of the nuclei but no phagocytosis.

TABLE 13.  
OPSONIN FOR B. WELCHII IN SERUM OF IMMUNIZED RABBIT

Incubation	Leukolysis	Phagocytosis
15 min.	Slight vac.	0.7
10 "	" "	7.5
10 "	" "	7.7
7 "	"	6.
5 "	"	6.
3 "	0	5.
15 " No serum, NaCl Sol.	0	0.5
15 " No serum, NaCl Sol.	0	0.0

The rabbit used in this experiment (Table 13) had received

three injections, extending over a period of 25 days, of 0.1 c.c., 0.5 c.c., and 0.5 c.c. of a 48-hour culture, the last 10 days previously.

The table shows rapid phagocytosis especially in those tubes incubated seven minutes and less. Evidently there was little or no leukocytolytic acid substance in this serum.

After heating the immune serum to 60° C. for 30 min. it gave but little phagocytosis.

**The Bactericidal Action of Immune Serum.**—For the study of lysis of *B. welchii* by rabbit and guinea-pig serum the method of Neisser and Wechsberg was followed:

Each tube contained 0.0002 c.c. of a 24-hour broth culture of the bacillus, 0.15 c.c. plain broth, the amount of serum indicated, and enough NaCl solution to make the total volume in each case 2.5 c.c. The tubes were incubated at 37° C. for three hours when agar plates were made, using 0.25 c.c. of the mixture for each plate. The plates were incubated for 24 hours under anaerobic conditions (H gas) at 37° C., when the number of colonies on each plate was estimated.

TABLE 14.  
THE EFFECT OF NORMAL RABBIT SERUM ON *B. WELCHII*.

Amount of Serum	Number of Colonies
0.5 c.c.	∞
0.25 "	∞
0.1 "	Thousands
0.05 "	Hundreds
0.025 "	Hundreds
0.01 "	Hundreds (Isolated)
0.005 "	∞
0.0025 "	∞
0.05 " (Heated to 56° C. 30 min.)*	∞
0.05 " " " "	Hundreds
0.05 " (No culture)	0
0.0 (Culture)	∞

∞ = Innumerable.

\* = Guinea-pig serum complement (0.3 c.c.).

Table 14 shows that normal rabbit serum is slightly bactericidal; and this action is most marked in mixtures containing 0.025 c.c. and 0.01 c.c. of serum. Under similar conditions 0.025–0.01 c.c. of normal guinea-pig serum is perhaps slightly bactericidal.

Table 15 gives the results obtained with the serum of rabbits immunized with *B. welchii*, heated to 56° C. for 30 minutes and complemented with normal guinea-pig serum. The rabbit serum

was obtained from animals that had received three injections of living cultures (0.1, 0.5, 0.5 c.c.) extending over a period of 20 days.

TABLE 15.

THE EFFECT OF IMMUNE RABBIT SERUM ON B. WELCHII.		
Amount of Heated Rabbit Serum	Amount of Guinea-Pig Serum	Number of Colonies
0.5	0.3	∞
0.25	"	∞
0.1	"	∞
0.05	"	Thousands
0.025	"	Thousands
0.01	"	Hundreds
0.005	"	51
0.0025	"	78
0.25	.0	∞
0	0.3	∞

∞ = Innumerable.

The antibacterial action begins to show itself in the mixtures containing 0.05 c.c. of rabbit serum and is most marked in those containing 0.005 c.c. and 0.0025 c.c. On comparison of Table 14 with Table 15 it is seen that the immune serum shows a slight increase in antibacterial power. The normal guinea-pig serum (0.3 c.c.) used as the complement is by itself without bactericidal action.

The maximum bactericidal action being caused by 0.01 to 0.005 c.c. of heated immune serum (amboceptor), it seemed advisable to use varying amounts of complement and a definite amount amboceptor to determine if 0.3 c.c. complement was the most efficient amount.

In the following series the amboceptor, culture, NaCl solution, and broth were mixed and placed at 37° C. for one hour when complement was added and the mixture incubated for two additional hours.

TABLE 16.

THE EFFECT OF IMMUNE RABBIT SERUM AND VARYING AMOUNTS OF GUINEA-PIG SERUM ON B. WELCHII.		
Amount of Heated Rabbit Serum	Amount of Guinea-Pig Serum	Number of Colonies
0.005	0.005	∞
"	0.01	∞
"	0.05	∞
"	0.1	∞ (slight reduction in no.)
"	0.2	70
"	0.3	65
"	0.4	Hundreds
"	.0	∞
0	0.5	∞

∞ = Innumerable.

Here again the maximum antibacterial action was obtained in mixtures of 0.005–0.0025 c.c. immune serum and 0.2–0.3 c.c. guinea-pig serum. The interesting phenomenon of complement deviation is well illustrated in Tables 15 and 16.

Evidently normal and immune rabbit serum at proper concentration is distinctly bactericidal for *B. welchii*. Microscopic examination shows the bacteria in the mixtures intact but dead, so that no growth could be obtained with them. No effect was obtained on animal inoculation. There was no evidence of any disintegration among the bacterial cells. The process is therefore evidently a bactericidal and not a bacteriolytic one.

**Agglutinins.**—According to Scheffler,<sup>1</sup> Clairmont and Rothbergers,<sup>2</sup> specific agglutinins are produced on immunization with *B. welchii*. Clairmont found the sera of animals immunized with one strain of *B. welchii* to agglutinate not only the homologous strain but also all strains coming from infant stools. Passini<sup>3</sup> found the agglutinins produced in some related antisera and in the homologous sera when different strains were used. Werner<sup>4</sup> secured an agglutinating serum which acted in dilutions of 1:1000.

Some observers, among them Kamen,<sup>5</sup> have been unable to demonstrate agglutinins in the sera of immunized animals.

The bacteria in my experiments were obtained from a 24-hour culture grown in 10 c.c. of broth under paraffin oil. The mixtures of bacteria and serum dilutions were incubated at 37° C. for varying periods. The presence or absence of agglutination was determined by naked eye at times assisted by microscopic examination.

The agglutinins begin to appear in the serum about the sixth or seventh day after injection of the heated (60° C. 30 min.) bacterial culture and increase until the 12th or 14th day. On reinoculation several times the agglutinins may be increased markedly. In the case of the serum of a rabbit receiving four injections of live bacteria (0.1, 0.3, 0.5, 0.5 c.c.) in the course of 24 days distinct agglutination was obtained in dilutions of 1:1200 after an incubation of three

<sup>1</sup> Cited by Paltauf, *Kolle u. Wassermann Handbuch*, 1904, 4, p. 706.

<sup>2</sup> *Ibid.*

<sup>3</sup> *Wien. klin. Wchnschr.*, 1905, 18, p. 921.

<sup>4</sup> *Arch. f. Hyg.*, 1904, 50, p. 274; 1905, 53, p. 128.

<sup>5</sup> *Centralbl. f. Bakt.*, 1904, 35, pp. 554–686.

hours. The maximum and most typical agglutination occurred at 1:500 dilution in  $2\frac{1}{2}$  hours. Not all rabbits produce agglutinins to the same extent and a serum agglutinating 1:1000 is rare.

**Precipitins.**—During the immunization of rabbits with *B. welchii* precipitins develop in the serum, as well as opsonins, bactericidal substances, and agglutinins. The precipitins appear about the seventh day and gradually increase until about the 12th–14th day when a gradual decline begins.

In order to demonstrate precipitins 24- and 48-hour broth cultures of *B. welchii* were filtered (Berkefeld); this filtrate contains quite a little organic acid which increases the speed of the reaction when the serum is added. For example, one rabbit, which had received four injections of 0.1, 0.3, 0.5, 0.5 c.c. of filtrates, was bled six days after the last injection and the serum contained typical precipitin. When the antiserum was brought in contact with the bacterial filtrate it required approximately 0.25 to 0.5 c.c. of serum to produce a permanent clouding in 10 c.c. of filtrate and it required approximately 7 to 10 c.c. of filtrate to produce a precipitation in 2.5 c.c. of antiserum. The reaction usually took place in from 10 to 15 minutes at room temperature and in 5 to 7 minutes in the incubator. A quantitative estimation of the precipitin, precipitinogen, and precipitate was not made.

#### ANAPHYLAXIS TO THE PROTEINS OF *B. WELCHII*.

Broth cultures of *B. welchii* were grown for several days and precipitated by centrifugalization. The precipitate was then ground in a sterile mortar and 0.01, 0.03, 0.04, 0.05 and 0.1 c.c. in sterile 0.85 per cent NaCl solution injected into five guinea-pigs respectively. In 15 days all these guinea-pigs were injected with 1.00 c.c. of a like emulsion in 0.85 per cent NaCl solution. All showed typical anaphylactic symptoms and one died. Controls injected with corresponding amounts of broth, as well as those receiving one injection of 1 c.c. of the emulsion, gave negative results. The relation of anaphylaxis to the partial immunity of the rabbit has not been investigated. It has been determined, however, that the rabbit can be sensitized in the same manner as the guinea-pig to the bacterial protein.



## SUMMARY.

*B. welchii* shows varying degrees of pathogenicity for rabbits even when no necrotic tissue exists in the body. The bacterium is highly pathogenic for guinea-pigs. Rabbits are far more resistant to infection than guinea-pigs. If necrotic tissue is present in the body *B. welchii* grows rapidly.

Rabbits injected intravenously with cultures of *B. welchii*, immediately killed and incubated for one hour, frequently show evidence of necrosis in the cells around the interlobular veins of the liver, demonstrating the very rapid action of the toxic substance.

*B. welchii* never produces gas in the systemic blood vessels of the body ante mortem. Rapid saccharo-butyric fermentation begins within one hour post mortem in some animals. If necrotic tissue is present in the body, fermentation and gas production may take place at this point at any time during life.

The toxic substances produced by *B. welchii* in dextrose broth cultures are not destroyed completely until the material has been heated to 100° C. for several hours. Beginning at 70° C., the toxic substances are volatilized or rendered inert and this takes place rather rapidly at 100° C. The decrease in the toxicity of the cultures in a measure corresponds to the extent of the volatilization or destruction. The toxic substances are not true bacterial toxins but secretory zymogenic products produced by the bacterial cells capable of generating organic acids. In infections in the body the acids produced act as toxic agents. Butyric acid of the same acidity as the cultures produces the same effect.

No distinct toxin could be isolated from *B. welchii* by the various methods. The organic acids produced increase the protein digestion which goes on in *B. welchii* infections.

Atypical hemolysis is produced by cultures of *B. welchii* and by the serum of rabbits for a short period after they have been injected with the bacterial culture. As organic acids cannot exist free in the blood, this effect of the serum is probably due to formation, by the acids produced, of salts which are not rapidly eliminated. The lytic agent eventually disappears from the blood. The hemolytic action of the cultures is due to the organic acids. Intravascular hemolysis does not occur in animals.

*B. welchii* produces leukocytotoxic substances, which also undoubtedly are organic acids.

Opsonins for *B. welchii* are present in normal rabbit serum and may be increased on immunization. Very little phagocytosis occurs unless the bacteria are washed free from organic acids because the organic acids neutralize the opsonins in the serum and cause marked changes in the leukocytes.

Normal and immunized rabbit serum is bactericidal for *B. welchii*. The serum of my animals was most effective in concentrations of amboceptor 0.01–0.005 c.c. and complement 0.3 c.c. (guinea-pig serum). Normal guinea-pig serum is only slightly destructive for *B. welchii* when compared with rabbit serum. The phenomenon of “complement deflection” occurs in the bactericidal action of rabbit and guinea-pig sera.

The action of the serum is purely bactericidal. There is no lysis.

Agglutinins develop in the sera of immunized rabbit. In one rabbit the serum agglutinated in a dilution of 1:1200, the optimum agglutination occurring at a dilution of 1:200 and 1:500. The agglutinins after several injections rise to the highest point in the serum about the 14th to 21st day, then gradually decline.

Precipitins are developed in the sera of immunized rabbits.

The proteins of *B. welchii* give the anaphylaxis reaction.

## THE INFLUENCE OF SCURVY ON HEMORRHAGES IN PLAGUE.\*

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(From the Laboratory of the United States Public Health and Marine Hospital Service, Oakland, California.)

It is sometimes stated that extensive hemorrhages in plague do not occur so frequently now as in some of the older epidemics. According to Scheube:<sup>1</sup> "In some epidemics especially they are so numerous that the whole body appears black, hence the designation of Black Death."

Hirsch<sup>2</sup> says with regard to this pandemic "of the middle of the fourteenth century, which, under the name of the Black Death came from the East and overran a large part of the old world": "It follows from the statements of all the chroniclers of this pestilence—medical and non-medical—that spitting of blood was one of the commonest phenomena in the course of the disease." It seems somewhat uncertain as to whether the designation Black Death was derived from the special prevalence of cutaneous hemorrhages, for Hirsch makes the significant statement that "unfortunately the available records of plague are poor in clinical facts; so that it is difficult to come to a definite opinion whether there are any considerable differences between the several epidemics as to the frequency of hemorrhages in general, or as to the particular kinds of hemorrhages."

However, it seems possible that the social misery of the fourteenth century was accompanied by the prevalence of scurvy, a disease which might well contribute to the degree of hemorrhages which occurs in the normal individual. Epidemic scurvy was not recognized by the medical profession until the fifteenth century. Hirsch says: "It is *a priori* highly probable that scurvy had been epidemic from time to time in antiquity under the same circumstances that have given rise to it in the modern period or in recent times. It certainly follows from the account given by Jacques de Vitry of a disease called by him the plague which ravaged the army of the Crusaders before Damietta in 1218, and from Joinville's description

\* Received for publication July 28, 1909.

<sup>1</sup> *The Diseases of Warm Countries*, Phila., 1903, p. 23.

<sup>2</sup> *Handbook of Geographical and Historical Pathology*, London, 1883-86, I, p. 540.

of the sickness that broke out in 1250 among the army of Louis IX at the siege of Cairo, that scurvy had existed long before we have any medical recognition or description of it as a peculiar form of disease."

I have noted from time to time that different guinea-pigs inoculated by the cutaneous method from different rats or from the same rat showed varying degrees of petechial or periglandular hemorrhages. Notes were kept on the variety of guinea-pigs used and in one series it seemed that the rough haired variety was more prone to hemorrhagic extravasations. However, further observations failed to substantiate that one. The probable explanation was not thought of until I read the "Experimental Studies, Relating to Ship-Beri-Beri and Scurvy" by Holst and Frölich.<sup>1</sup> Their work showed that when guinea-pigs of 300-600 gm. were kept on a "one sided" diet consisting of various sorts of grain, groats, and bread, they died in 18 days or more from a disease that corresponded, macro- as well as microscopically, to human scurvy. They also proved the favorable influence of various "anti-scorbutics" the value of which has been demonstrated by human experience, such as cabbage, and fresh potatoes. A "one-sided" diet of the latter vegetables failed to produce the disease.

I have confirmed their observations as far as most of the gross pathological changes are concerned, altho my naked-eye examination of the osseous system has not been nearly as thorough. Their experiments clearly demonstrate that guinea-pigs rapidly become abnormal when kept on certain single diets and it seems as if these facts should be borne in mind in keeping these animals for experimental purposes. Before reading their paper, I encountered stock guinea-pigs on two occasions which died of general anasarca with muscular hemorrhages, cases which showed no bacteria on microscopic examination and which now appear to have been guinea-pigs with scurvy.

Experiments were undertaken to determine the influence of scurvy on the general course of plague in guinea-pigs with some attempt at determining whether a salt antagonism existed or not. However, the experiments reported here are believed to be merely suggestive and not at all conclusive. A preliminary experiment was conducted as follows:

<sup>1</sup> *Jour. Hyg.*, 1907, 7, p. 634

Two guinea-pigs, whose initial weight was 385 and 340 gm. respectively, which had been kept on a diet of rolled barley and water only for 17 days were inoculated cutaneously with spleen tissue from a guinea-pig dead of acute rat plague and were then kept on a diet of barley and water only. They both died in about 48 hours and on dissection showed buboes which were very much more hemorrhagic than usual in guinea-pigs and the large hemorrhagic extravasations about the buboes extended up along the posterior wall of the pelvis and abdomen. The adrenals were injected and hemorrhagic. There were a few subserous petechiae on the intestines (not unusual). The mesenteric glands showed subcapsular hemorrhages. There were a few small hemorrhages about the knee joints. The blood escaping from the vessels was not coagulated in 15 minutes. Microscopically there was a marked septicemia with rods resembling *B. pestis*.

Further experiments were conducted as follows:

**Series 1.** Ten adult guinea-pigs were kept supplied with a liberal amount of rolled barley and water. On the 23rd day two, Nos. 2 and 5, died of scurvy with a loss in weight of 249 and 170 gm. respectively. No. 2 showed areas of congestion and grayish discoloration about the roots of the teeth of the lower jaw; a few petechial hemorrhages about the axillary and inguinal glands and into the capsule of the glands, and some diffuse extravasations about the knee and elbow joints; the molar teeth could be removed with ease and their roots were blood stained. No. 5 showed the same changes but the hemorrhages were more marked. On the next day (24th) the remaining pigs were inoculated cutaneously with plague cultures, Nos. 1-8, and a control normal stock, No. 11, with a 24-hour old culture of virulent rat plague which had been isolated three months before; and Nos. 9, 10, and a control normal stock guinea-pig, No. 12, were inoculated cutaneously with an avirulent plague culture "Bombay."

As most of the animals were in a very weak condition they were kept after inoculation on a diet of cabbage and carrots. Within 24 hours most of them had gained in strength visibly.

Table 1, p. 568, shows the loss in weight, time till death, and the anatomical findings in the series.

**Series 2.** Ten adult guinea-pigs were kept in the same manner as those in Series 1, but in addition received daily 8 gm. of  $\text{CaCl}_2$  dissolved in their drinking water. On the 24th day No. 10 whose initial weight was 388 gm. died; it weighed 235 gm. and on dissection showed none of the periglandular and periarticular hemorrhages seen in the animals kept on barley and water alone, but its teeth came out readily and there were small hemorrhages into the axillary and inguinal glands. The remaining nine animals were very much stronger and livelier than those kept on barley and water only. Numbers 1-6 were inoculated cutaneously with the virulent culture of rat plague used in Series 1 and at the same time the animals in the first series were inoculated; and Nos. 7-9 with the avirulent culture "Bombay." They were fed on cabbage and carrots after the inoculation.

Table 2, p. 569, shows the results of these inoculations.

In neither series did the guinea-pigs inoculated with the avirulent culture "Bombay" contract plague.

The degree of hemorrhage which occurred in guinea-pigs 3 and 8,

<sup>1</sup> See McCoy, *Jour. Infect. Dis.*, 1900, 6, p. 170.



Series 1, and 6, Series 2, was very much greater than I have ever seen in guinea-pigs without scurvy. This is also true concerning the guinea-pigs used in the preliminary experiment. Had the animals in Series 1 and 2 been inoculated a few days sooner and then been kept on barley and water the results might have more nearly approximated than those obtained in the preliminary experiments.

#### SUMMARY AND CONCLUSIONS.

Scorbutic guinea-pigs when inoculated with plague may show a greater degree of hemorrhagic extravasation post mortem, than is usually seen. In a single experiment with two animals, which were kept on barley and water for 17 days, then inoculated with plague and kept on the scorbutic diet, death occurred in about 48 hours; and the coagulation of the blood was seen to be delayed for more than 15 minutes. In another series where six guinea-pigs were kept on barley and water for 24 days they became so weak that they were fed antiscorbutics, cabbage and carrots, after inoculation with plague; and only two of these animals showed a degree of hemorrhage which could be considered excessive for either plague or scurvy in the guinea-pig.

When six guinea-pigs, kept on the same scorbutic diet for 24 days, received calcium chloride in their drinking water, they kept in better health and only one showed excessive hemorrhage when inoculated with plague.

These experiments are not conclusive but suggestive in showing that there might be excessive hemorrhage when plague and scurvy occur in the same individual.

TABLE 1.

No.	Initial Weight in Grams	No. of Days till Death after Inoculation	Weight in Grams at Time of Inoculation	Loss in Weight in Grams	Remarks
1.....	630	6	515	115	Chloroformed in a sickly condition; large slightly congested buboes; large speckled spleen; numerous consolidated areas, 2-3 mm. in diameter, in lungs.
3.....	530	4	440	90	Good general condition; marked local reaction at left of median line; sero-gelatinous exudate over abdomen; large left inguinal bubo surrounded by very marked periglandular hemorrhages; intestines covered with petechiae; spleen large and soft and full of foci of necrosis; liver congested and full of foci of necrosis; lungs normal; left pelvic glands enlarged and deeply congested; right inguinal gland of normal size but slightly congested, a few small petechiae in fascia about right knee joint, none about left knee joint.
4.....	640	- 1	340	300	Not infected; marked scurvy; numerous diffuse subcutaneous and cutaneous hemorrhages 6 to 12 mm. in diameter about sides and back.
6.....	510	- 1	325	185	Not infected; marked scurvy; small hemorrhages in the skin and about knee joints.
7.....	460	2.5	287	173	No local reaction; small left inguinal bubo with slight periglandular hemorrhage; secondary left pelvic bubo with numerous capsular petechiae; numerous petechiae in both axillary regions and under serosa of lungs; spleen large, soft, and deeply congested; large sub-facial hemorrhages about thighs and knee joints.
8. ....	481	4.5	328	153	Good local reaction; cutaneous hemorrhages 3 mm. in diameter about an inch distant from local reaction; large left inguinal bubo with very marked hemorrhagic extravasations which extend down over left knee joint; large irregularly shaped hemorrhagic extravasation in right axilla; spleen large and speckled; liver congested; lungs pale and show a few petechiae; intestines covered with petechiae; slight serous ascites.
11 control	441	4.5	441	...	Kept on normal diet of cabbage and barley until inoculation; periglandular hemorrhage almost as marked as in No. 8, but not as marked as in No. 3; no hemorrhages away from bubo.

TABLE 2.

No.	Initial Weight in Grams	No. of Days till Death after Inoculation	Weight in Grams at Time of Inoculation	Loss in Weight in Grams	Results
1.....	461	4.5	358	103	Good local reaction; left inguinal bubo not very large but well congested; good periglandular exudate; spleen and liver speckled.
2.....	519	3 ±	366	153	Small but typical local reaction with small cutaneous hemorrhages; good right inguinal bubo surrounded by marked hemorrhages; small deeply congested left inguinal bubo; spleen large, soft, and full of foci of necrosis; quite marked hemorrhagic extravasations about both knee joints; liver deeply congested.
3.....	564	4.5	462	102	Typical plague but not much periglandular hemorrhage; no hemorrhage about joints; in good physical condition.
4.....	505	3 ±	523	+	In better general condition than most of the others; marked local reaction and marked hemorrhage about bubo; serous ascites; no hemorrhages about knee joints.
5.....	426	3 ±	317	109	No local reaction; double buboes surrounded by marked hemorrhages; no hemorrhages about the knee joints.
6.....	475	3 ±	358	117	Marked local reaction; whole left bubo and groin is filled by a large hemorrhage, about 2 inches in diameter, and hemorrhages extend down the left thigh and about the knee joint; the hemorrhage on the left side extends into the pelvis and the left pelvic gland is intensely hemorrhagic; numerous petechiae over intestines; few about right knee joint.



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## THE FECAL BACTERIA OF HEALTHY MEN.\*

### PART II. QUANTITATIVE CULTURE EXPERIMENTS.

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CONCLUSIONS.

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#### INOCULATION MATERIAL.

THE homogeneous suspension of the mixt feces, containing one part of feces in one hundred parts of suspension, was employed as the source of material † for all the culture experiments. This original

\* Received for publication August 8, 1909.

† See Part I, this *Journal*, 1909, 6, p. 126.



suspension was called No. 1. One c.c. of it contained the bacteria of 10 mg. of the fresh feces. From it a series of dilutions was prepared and numbered as follows:

Suspension No. 2, 1 to 1,000, 1 c.c. represents 1 mg. feces.

Suspension No. 3, 1 to 10,000, 1 c.c. represents 0.1 mg. feces.

Suspension No. 4, 1 to 100,000, 1 c.c. represents 0.01 mg. feces.

Suspension No. 5, 1 to 1,000,000, 1 c.c. represents 0.001 mg. feces.

Each suspension was thoroughly mixt as prepared, and again each time before measuring out portions from it. A portion of Suspension No. 2 was transferred to a test tube and heated in a water bath at 80° C. for 15 minutes and then cooled. By this means the vegetative bacteria were killed and the resulting inoculation material was designated as Suspension No. 2, Spores. This procedure of quantitative dilution of the feces we consider to be of special value in making cultures, as it is then possible to estimate the number of cultivable bacteria demonstrated by any particular culture method upon a definite quantity of the original feces. The results of cultures thus become quantitatively comparable one with another and also with the direct microscopic examinations. It seems to us very desirable that quantitative methods of this kind should be generally adopted and employed in estimating the importance of any species in the fecal flora.

The variety of bacteria which may occur in the human feces is almost endless, but the species of real significance are limited in number. The accidental, unimportant bacterial species are represented by relatively few individuals, but may assume a very prominent place in cultures made without regard to quantitative methods. Even among the really significant members of the fecal flora, quantitative culture methods will be necessary to determine the real importance of the various species. In the classical work upon fecal bacteria it has generally been assumed that the relative importance of the different species in artificial cultures is a direct indication of their relative importance in the feces. The fallacy of this assumption is illustrated by the work of Escherich<sup>5</sup> and of Tissier<sup>17</sup> on the fecal flora of the breast fed infant. Thus Escherich, studying the fecal flora of the nursling by the gelatin plate method, concluded that *B. lactis aerogenes* and *B. coli* make up practically the entire bacte-

rial content of the breast fed infant's stool. Tissier, employing a different culture method, deep tubes of glucose agar, concluded that an entirely different microorganism, *B. bifidus communis*, makes up practically the whole flora of the breast fed infant's stool. Each of these observers carried out most careful direct microscopic examinations of the feces in conjunction with his culture experiments and found the bacteria cultivated to be morphologically identical with those in the original material. Tissier's work following that of Escherich clearly disproves Escherich's conclusion, but it is not impossible that some new culture method may bring to development a new species of bacteria occurring in still greater quantity in the nursling's stool than even *B. bifidus*. Tissier's conclusion that the species giving rise to the greatest number of colonies in deep glucose agar tubes is identical with the dominant microorganism seen microscopically in the nursling's feces, even though rendered probable by the similarity of the two in morphology and staining properties, will be made certain only when it is shown that the number of colonies of this bacillus, developed from a definite quantity of feces, bears some approximate relation to the number of bacilli of this type counted microscopically in the same quantity of the feces. Escherich clearly recognized the source of error in interpreting the results of culture experiments and the discrepancy between them and the results of direct microscopic examination. Thus he\* says: "Dabei stellten sich alsbald wesentliche Unterschiede in dem Ergebniss beider Untersuchungsmethoden heraus in der Art, dass die Zahl und Mannigfaltigkeit der im microscopischen Bilde vorhandenen Bakterien eine erheblich grössere war als diejenige der in der Cultur erhaltenen. Trotz aller Variationen des Nährbodens blieb das Resultat das gleiche und ich musste mir sagen, dass wenigstens mit den von mir ausschliesslich angewandten Methoden des festen Nährbodens das Ergebniss der Cultur nur im *positiven*, *niemals im negativen* Sinne entscheidend sein könne. Das microscopische Bild wird uns den festen, objectiven Rahmen liefern müssen, in welchen wir die zunächst noch unvollständigen Ergebnisse der Culturmethoden einzureihen haben." Tissier also observed bacterial forms in his microscopic preparations of feces which he was unable to bring to development

\* *Darmbakterien des Säuglings*, p. 12.

in culture, altho in some places he speaks of the deep glucose agar tube as a universal culture method.\*

The postulate so clearly enunciated by Escherich is certainly the safe rule. It does not seem probable that any single culture method will ever be devised which will bring to development all the living microorganisms present in the human feces, or even be equally favorable to the several different species which may grow upon it. More definite and certain progress will be made by culture methods which are frankly recognized as bringing to development certain definite species or groups of species to the exclusion of others, applied in a quantitative manner, the results of which may be directly compared with each other and with the results of direct microscopic examination of the original material. The results of our culture experiments are essentially incomplete and having been carried out quantitatively this incompleteness is clearly evident. We consider them therefore of peculiar value in connection with the direct quantitative observations recorded in Part I, with which they may be quantitatively compared.

#### PLATE CULTURES.

Three sets of plate cultures were made from the unheated material: (1) upon litmus lactose agar at 37° C. in the presence of air, (2) upon litmus glucose agar at 37° C. in an atmosphere of hydrogen, and (3) upon litmus lactose gelatin at 18° C. in the air. In preparing these plates, portions of 0.25 c.c. of Suspension 3, and 0.50 c.c. of Suspensions 4 and 5 were measured out into sterile Petri dishes. The agar was melted and cooled to 45° C. in a constant temperature water bath. Five drops of a sterile strong solution of purified litmus† were added to each tube of medium, the contents of the tube then added to the bacterial suspension in the Petri dish, covered, and thoroughly mixt, and then allowed to solidify in a level place. The gelatin was also colored with the litmus after it was melted ready to pour into the Petri dishes. When solidified, the lactose agar plates were transferred to the incubator and allowed to develop for 24 hours. The colonies on each plate were then counted and the number of bacteria

\* "Elle est en effet absolument générale," Thèse, p. 41; and "Si nous avons insisté sur cette méthode, d'isolement c'est qu'elle nous paraît absolument générale," Thèse, p. 43.

† We have found Merck's Highest Purity Litmus to be satisfactory.

per milligram feces represented by them estimated. The glucose agar plates, after solidification, were placed in a Novy anaerobe jar, stacked upon a low glass tripod (see Fig. 1). Upon the bottom of the jar, underneath the plates, a small amount (about 2 gm.) of pyrogalllic acid was placed, and on top of the stack of plates, a small (50 c.c.) flask filled with strong sodium hydroxide solution. The flask (see Fig. 2) was provided with a syphon spout to the end of which a rubber tube was attached, leading down at the side of the stack of Petri dishes to the bottom of the jar. A small tube containing glucose gelatin, colored with a few drops of methylene blue and then steamed for a short time, was also placed in the jar to serve as an indicator control of the anaerobiosis, the leuko-methylene blue

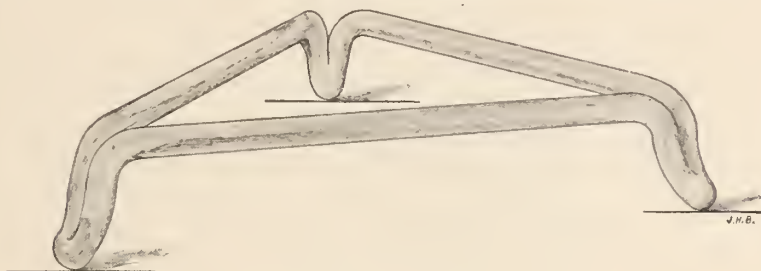


FIG. 1

remaining reduced and colorless in the absence of oxygen. The cover of the jar was then clamped on, and hydrogen passed through it for several hours. The stop-cock was then closed, the jar disconnected from the hydrogen generator, and inclined slightly to start the flow of alkali through the syphon spout from the flask down onto the pyrogalllic acid in the bottom of the jar. In this way the pyrogalllic acid became available for absorption of the last traces of oxygen. The jar was then placed in the incubator at 37° C. for 48 hours, after which the colonies were counted and the bacteria per milligram feces calculated. The gelatin plates were allowed to develop for four days at 18°-20° C., after which the colonies were counted and the bacteria per milligram feces calculated. The reaction of the colonies on the litmus medium was noted in each case. On the lactose (aerobic) agar, alkaline colonies were frequently observed, generally corresponding in number to the alkaline colonies develop-

ing on the gelatin plates from the same stool. All the colonies developing on the anaerobic glucose agar plates were invariably acid in reaction, but from time to time colonies producing an unusually intense red color were noted. The gelatin plates permitted an immediate separation of the colonies into four kinds, according to reaction and liquefaction, as well as producing more definitely recognizable types of colonies. The relative numbers of different colonies were noted here in addition to the total colonies.

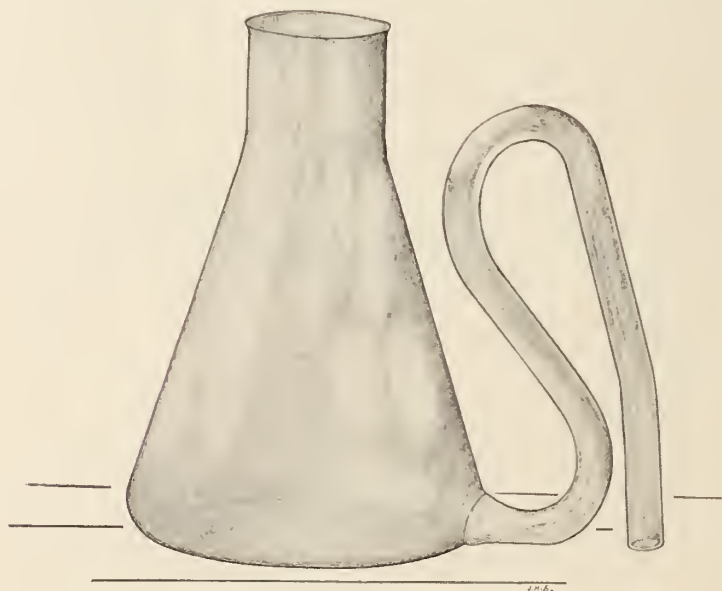


FIG. 2

Table 8 shows the number of colonies developed on the aerobic agar and anaerobic agar plates from 202 different stools, and the number of colonies developed on the gelatin plates from 144 stools, and in these latter the relative numbers of liquefying and non-liquefying, acid and alkaline colonies. These results present nothing new in themselves, being essentially the same as obtained by a large number of investigators. They are, however, of particular importance in connection with the other examinations made upon the same stools, especially the direct quantitative examinations reported in Part I.



The number of colonies on these three sets of plate cultures, aerobic litmus lactose agar, anaerobic litmus glucose agar, and aerobic litmus lactose gelatin, generally in close agreement, represents approximately the quantity of cultivable *B. coli* in the feces. Other bacteria occurred in small numbers and not constantly. The smallest number of colonies per milligram feces was observed in Subject G, Dec. 14, 1,360 on the aerobic agar, but 6,440 on the anaerobic agar. Subject D, April 27, shows 2,420 on the aerobic agar, 2,900 on the anaerobic agar, and 3,140 on the gelatin plates. The total number of bacteria by direct microscopic counts upon these stools was 179,000,000 and 252,000,000 bacteria per milligram respectively. The highest colony count occurs in Subject F, June 8, 1,500,000\* per milligram on the aerobic agar plates. The anaerobic agar plates show 1,100,000, but the gelatin plates only 380,000 per milligram. The direct count on this sample of feces was 477,000,000 bacteria per milligram. Subject E, Mar. 2, shows 1,200,000 colonies per milligram on all three sets of plates. The direct count in this case was 480,000,000 bacteria per milligram. Of the 12 individuals, Subject E shows the highest average value, 280,000 colonies per milligram feces, Subject J, the lowest, 33,000. The average of the direct counts for these subjects were 424,000,000 and 328,000,000 bacteria per milligram feces. There is moreover a remarkable variation in the number of cultivable *B. coli* in different stools of the same individual, and this variation seems to bear no constant relation to the total number of bacteria present, nor to the dryness of the stool, altho these factors appear to have an influence in some cases. In Table 9, the maximum, mean, and minimum number of colonies developed on the plates, and of bacteria counted directly, are given, summarized from Tables 8 and 2. The extreme individual variations are here clearly shown.

Sucksdorff,<sup>15</sup> who first noted this remarkable variation in the number of cultivable bacteria in the human feces, ascribed it to the variation in the number of bacteria swallowed with the food. By taking only sterilized food he reduced very considerably the number of colonies developed from the feces. Very serious errors in Sucksdorff's

\* Attention should be called to the fact that inasmuch as the same dilutions were employed in making the plates from each stool, these high counts are really too low, because of inhibition of development by crowding on the plates.

TABLE 8.  
BACTERIA PER MILLIGRAM FECES, DEVELOPED INTO COLONIES ON PLATE CULTURES.  
SUBJECT A

LABORATORY No.	DATE	UNHEATED SUSPENSION										SPORE MATERIAL (HEATED AT 80° C. 15 MINUTES)			MICROSCOPIC COUNT
		Aerobic Agar	Anaerobic Agar	Aerobic Gelatin						Aerobic Agar	Anaerobic Blood Agar				
				Total Colonies	Bacteria				Molds P. ct.		Colonies	Types			
					Non-liq. Acid P. ct.	Liquefying Acid. P. ct.	Liquefying Alk. P. ct.								
B 37.....	Nov. 12	17,400	10,000	.....	.....	.....	.....	.....	..	.....		336,000,000			
B 60.....	Nov. 26	7,100	10,000	.....	.....	.....	.....	.....	..	.....		300,000,000			
B 83.....	Dec. 10	20,200	21,100	.....	.....	.....	.....	.....	..	.....		358,000,000			
B 110.....	Dec. 16	21,200	27,000	6,400	.....	.....	.....	.....	..	.....		244,000,000			
B 122.....	Jan. 6	15,600	16,700	100.0	0.0	0.0	0.0	0.0	..	.....		339,000,000			
B 134.....	Jan. 22	52,680	51,322	100.0	0.0	0.0	0.0	0.0	..	.....		348,000,000			
B 146.....	Feb. 6	16,780	16,780	100.0	1.0	0.0	0.0	0.0	..	4,407	I	447,000,000			
B 158.....	Mar. 11	59,609	31,766	100.0	0.0	0.0	0.0	0.0	..	915	I	472,000,000			
B 170.....	Mar. 27	19,720	18,020	100.0	0.0	0.0	0.0	0.0	12	632	I-II	407,000,000			
B 182.....	April 10	14,620	14,380	100.0	0.0	0.0	0.0	0.0	23	counted	I-II-III	478,000,000			
B 104.....	April 27	38,674	30,800	100.0	1.0	0.0	0.0	0.0	7	97	I-II	478,000,000			
B 206.....	May 13	64,628	37,771	100.0	0.0	0.0	0.0	0.0	4	367	I-IV	359,000,000			
B 218.....	May 28	20,000	57,818	100.0	0.0	0.0	0.0	0.0	17	8,467	I-III-VIII	539,000,000			
B 230.....	June 15	52,445	57,524	100.0	0.0	0.0	0.0	0.0	6	345	I-II-III	472,000,000			
B 242.....	July 1	47,861	51,309	100.0	0.0	0.0	0.0	0.0	24	309	III-V-VIII	347,000,000			
B 254.....	July 17	94,691	57,542	100.0	0.0	0.0	0.0	0.0	46	13,608	I-III	441,000,000			
B 266.....	July 31	57,200	55,763	Plates not counted					3,556	6,625	I-VI	394,000,000			
Average.....		35,632	31,583	99.30	0.70	0.00	0.00	0.00	411	3,595		304,000,000			
Minimum.....		7,100	10,000	97.00	0.00	0.00	0.00	0.00	4	97		244,000,000			
Maximum.....		94,691	57,542	100.0	3.00	0.00	0.00	0.00	3,556	13,608		539,000,000			

SUBJECT B.

[illegible]

SUBJECT C.

[illegible]

TABLE 8—Continued.  
SUBJECT D

LABORATORY NO.	DATE	UNHEATED SUSPENSION										SPORE MATERIAL (HEATED AT 80° C. 15 MINUTES)		MICROSCOPIC COUNT
		Aerobic Agar	Anaerobic Agar	Aerobic Gelatin						Total Colonies	Anaerobic Blood Agar			
				Bacteria	Molds		Aerobic Agar	Colonies	Types					
					Non-liq. Acid P. ct.	Liquefying A. fl. Alk. P. ct.					P. ct.			
B 34	Nov. 11	15,000	20,000	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	348,000,000
B 57	Nov. 23	70,000	58,000	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	448,000,000
B 80	Dec. 10	14,000	15,000	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	280,000,000
B 90	Dec. 23	8,007	10,200	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	387,000,000
B 123	Jan. 25	23,200	20,520	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	262,000,000
B 135	Feb. 10	7,620	8,000	100.0	0.0	0.0	0.0	0.0	0.0	27,080	.....	.....	.....	288,000,000
B 147	Feb. 27	38,740	42,010	100.0	0.0	0.0	0.0	0.0	0.0	7,720	.....	.....	.....	270,000,000
B 157	Mar. 11	6,080	6,500	98.0	2.0	0.0	0.0	0.0	0.0	38,160	.....	.....	.....	352,000,000
B 160	Mar. 27	13,560	12,020	100.0	0.0	0.0	0.0	0.0	0.0	3,090	.....	.....	.....	322,000,000
B 181	April 10	37,440	38,780	99.0	0.0	0.0	0.0	0.0	0.0	13,020	.....	.....	.....	308,000,000
B 193	April 27	2,420	2,000	99.0	1.0	0.0	0.0	0.0	0.0	40,600	.....	.....	.....	252,000,000
B 205	May 13	15,800	10,460	100.0	0.0	0.0	0.0	0.0	0.0	3,140	.....	.....	.....	338,000,000
B 217	May 28	32,080	34,000	100.0	0.0	0.0	0.0	0.0	0.0	15,480	.....	.....	.....	330,000,000
B 220	June 15	15,840	14,540	100.0	0.0	0.0	0.0	0.0	0.0	30,200	.....	.....	.....	265,000,000
B 241	July 1	74,000	61,020	100.0	0.0	0.0	0.0	0.0	0.0	17,360	.....	.....	.....	103,000,000
B 253	July 17	41,740	35,340	100.0	0.0	0.0	0.0	0.0	0.0	87,135	.....	.....	.....	307,000,000
B 265	July 31	36,400	28,800	99.0	0.0	0.0	0.0	0.0	0.0	41,200	.....	.....	.....	304,000,000
				99.0	0.0	0.0	0.0	0.0	0.0	36,378	.....	.....	.....	
Average		26,637	24,609	99.67	0.24	0.01	0.00	0.08		27,050	.....	.....	.....	395,000,000
Minimum		2,420	2,000	98.00	0.00	0.00	0.00	0.00		3,140	.....	.....	.....	103,000,000
Maximum		74,000	61,020	100.00	2.00	0.10	0.00	1.00		87,135	.....	.....	.....	448,000,000

SUBJECT E.

B 41	Nov. 14	113,050	102,000	.....	.....	.....	254,000,000
B 64	Nov. 27	130,000	147,000	.....	.....	.....	270,000,000
B 87	Dec. 13	170,000	130,000	.....	.....	.....	305,000,000
B 106	Dec. 30	153,000	171,000	.....	.....	.....	431,000,000
B 118	Jan. 16	151,000	231,000	.....	.....	.....	454,000,000
B 130	Jan. 31	30,000	36,000	.....	.....	.....	491,000,000
B 142	Feb. 19	221,833	105,236	100.0	0.0	0.0	498,000,000
B 149	Mar. 2	1,200,213	1,232,677	100.0	0.0	0.0	308,000,000
B 161	Mar. 16	449,231	302,066	100.0	0.0	0.0	486,000,000
B 173	Apr. 1	123,431	117,097	100.0	0.0	0.0	290,000,000
B 185	Apr. 16	200,020	224,423	100.0	0.0	0.0	535,000,000
B 197	May 1	142,312	133,176	100.0	0.0	0.0	541,000,000
B 209	May 18	90,310	137,144	100.0	0.0	0.0	514,000,000
B 221	June 3	833,591	836,115	100.0	0.0	0.0	513,000,000
B 233	June 30	232,271	269,281	100.0	0.0	0.0	343,000,000
B 245	July 6	96,864	100,860	100.0	0.0	0.0	468,000,000
B 257	July 22	299,674	322,168	99.0	0.0	1.0	373,000,000
Average		270,210	268,963	99.92	0.00	0.08	509,000,000
Minimum		30,000	36,000	99.00	0.00	0.00	424,000,000
Maximum		1,200,213	1,232,677	100.00	0.00	1.00	590,000,000

SUBJECT F.

[illegible]

\* Represented by a single colony.



TABLE 8—Continued.  
SUBJECT G.

LABORATORY NO.	DATE	UNHEATED SUSPENSION										STORE MATERIAL (HEATED AT 80° C. 15 MINUTES)		MICROSCOPIC COUNT
		Acrobic Agar	Anaerobic Agar	Acrobic Gelatin						Acrobic Agar	Anaerobic Blood Agar			
				Total Colonies	Bacteria			Molds						
					Non-liq. Acid P. ct.	Alk. P. ct.	Liquefying Acid. P. ct.				Alk. P. ct.			
B 42.....	Nov. 15	73,000	92,000	.....	.....	.....	.....	.....	.....	.....	.....	.....	208,000,000	
B 65.....	Nov. 29	1,800	16,000	.....	.....	.....	.....	.....	.....	.....	.....	.....	341,000,000	
B 88.....	Dec. 14	1,300	6,440	.....	.....	.....	.....	.....	.....	.....	.....	.....	179,000,000	
B 107.....	Jan. 2	6,100	19,500	.....	.....	.....	.....	.....	.....	.....	.....	.....	327,000,000	
B 119.....	Jan. 20	10,220	19,940	.....	.....	.....	.....	.....	.....	.....	.....	.....	260,000,000	
B 131.....	Feb. 3	5,120	9,840	7,040	98.0	2.0	0.0	0.0	0.0	0.0	621	.....	370,000,000	
B 143.....	Feb. 21	228,358	277,841	241,633	100.0	0.0	0.0	0.0	0.0	0.0	283	.....	534,000,000	
B 152.....	Mar. 4	51,070	40,350	56,827	99.7	0.1	0.1	0.1	0.1	0.1	2,010	.....	430,000,000	
B 164.....	Mar. 19	11,306	11,020	9,720	96.3	3.0	0.7	0.0	0.0	0.0	262	.....	534,000,000	
B 176.....	April 3	60,688	46,023	69,424	99.0	0.0	0.0	0.1	0.0	0.0	1,240	.....	370,000,000	
B 188.....	April 20	145,314	106,600	133,309	100.0	0.0	0.0	0.0	0.0	0.0	11	1,483	474,000,000	
B 200.....	May 4	27,173	31,865	25,180	99.0	1.0	0.0	0.0	0.0	0.0	2	1,035	468,000,000	
B 212.....	May 20	270,878	165,048	215,078	100.0	0.0	0.0	0.0	0.0	0.0	6	1,035	440,000,000	
B 224.....	June 5	100,404	86,072	90,288	100.0	0.0	0.0	0.0	0.0	0.0	14	2,009	407,000,000	
B 236.....	June 22	259,468	289,575	260,368	100.0	0.0	0.0	0.0	0.0	0.0	24	64,308	416,000,000	
B 248.....	July 9	275,179	292,692	295,000	100.0	0.0	0.0	0.0	0.0	0.0	30	319	483,000,000	
B 264.....	July 29	170,114	162,327	142,400	90.0	0.0	0.0	0.0	0.0	0.0	280	204	583,000,000	
Average.....		101,097	93,231	121,363	98.57	0.51	0.07	0.02	0.83	48	6,401	.....	400,000,000	
Minimum.....		1,300	6,440	7,040	90.00	0.00	0.00	0.00	0.00	2	204	.....	170,000,000	
Maximum.....		270,878	289,575	260,368	100.00	3.00	0.70	0.10	10.00	280	64,308	.....	583,000,000	



TABLE 8—Continued.  
SUBJECT J.

LABORATORY No.	DATE	UNHEATED SUSPENSION :										SPORE MATERIAL (HEATED AT 86° C. 15 MINUTES)		MICROSCOPIC COUNT
		Aerobic Agar	Anaerobic Agar	Aerobic Gelatin						Aerobic Agar	Anaerobic Blood Agar			
				Total Colonies	Bacteria			Molds			Colonies	Types		
					Non-liq. Acid P. ct.	Liquefying Acid. P. ct.	Liquefying Alk. P. ct.	P. ct.						
B 31	Nov. 9	2,000	6,687	.....	.....	.....	.....	.....	.....	.....	.....	.....	158,000,000	
B 54	Nov. 21	2,720	6,300	.....	.....	.....	.....	.....	.....	.....	.....	.....	322,000,000	
B 77	Dec. 7	62,400	36,500	.....	.....	.....	.....	.....	.....	.....	.....	.....	270,000,000	
B 100	Dec. 23	6,026	0,640	.....	.....	.....	.....	.....	.....	.....	.....	.....	268,000,000	
B 111	Jan. 9	Plates lost	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	208,000,000	
B 124	Jan. 25	40,000	41,000	.....	99.8	0.2	0.0	0.0	0.0	.....	.....	.....	300,000,000	
B 136	Feb. 10	12,000	14,586	.....	98.0	0.0	0.0	0.0	2.0	.....	.....	.....	220,000,000	
B 148	Feb. 27	22,358	31,182	.....	99.9	0.0	0.1	0.0	0.0	.....	.....	.....	386,000,000	
B 159	Mar. 13	15,813	10,626	.....	Plates lost			by melting	.....	.....	.....	.....	304,000,000	
B 171	Mar. 30	88,003	86,515	.....	100.0	0.0	0.0	0.0	0.0	.....	.....	.....	401,000,000	
B 183	April 14	34,420	28,700	.....	100.0	0.0	0.0	0.0	0.0	.....	.....	.....	430,000,000	
B 195	April 20	66,449	31,621	.....	100.0	0.0	0.0	0.0	0.0	.....	.....	.....	302,000,000	
B 207	May 15	2,320	3,240	.....	Plates lost			by melting	.....	.....	.....	.....	383,000,000	
B 219	June 1	66,375	40,410	.....	100.0	0.0	0.0	0.0	0.0	.....	.....	.....	302,000,000	
B 231	June 17	31,100	26,680	.....	99.4	0.3	0.3	0.0	0.0	.....	.....	.....	470,000,000	
B 243	July 4	5,520	4,900	.....	96.0	0.0	0.0	0.0	0.0	.....	.....	.....	368,000,000	
B 255	July 20	67,387	55,300	.....	99.8	0.0	0.0	0.0	0.2	.....	.....	.....	372,000,000	
Average	.....	32,117	27,501	.....	99.11	0.06	0.05	0.25	0.53	.....	.....	.....	328,000,000	
Minimum	.....	2,320	3,240	.....	96.00	0.30	0.00	0.00	0.00	.....	.....	.....	188,000,000	
Maximum	.....	88,003	86,515	.....	100.00	0.30	0.30	2.00	4.00	.....	.....	.....	470,000,000	

SUBJECT K.

[illegible]

SUBJECT L.

[illegible]

technic have been pointed out by Stern,<sup>14</sup> who has shown that the former's conclusions are not justified. From the results given in Tables 8 and 9, it is evident that the number of cultivable facultative anaerobes in the feces is not a reliable indication of the extent of bacterial multiplication in the digestive tract. This question will be more fully discussed below.

From the fecal suspensions heated to 80° C. for 15 minutes, two sets of plates were made, for aerobic spores and for anaerobic spores. Ordinarily quantities of 0.25 and 0.50 c.c. of Suspension No. 2, Spores, were plated in litmus lactose agar aerobically. Ninety-seven such examinations for aerobic spores are tabulated. Bacteria of this class were nearly always present in sufficient numbers to appear on these plates (0.5 c.c. of Suspension No. 2 represents 0.0005 gm. feces). Thin spreading colonies with alkaline reaction, characteristic in appearance, were observed 94 times in the 97 examinations. It is probable that this species is a normal component of the human intestinal flora, and for this reason it has been subjected to more detailed studies\* which have shown it to be a member of the *Urobacillus* group. The number of aerobic spores varied from 3,556 per milligram in Subject A, July 31, to 0 in Subject J, Apr. 29, the only examination in which growth was entirely absent from these plates. The average of the 97 examinations was 65 aerobic spores per milligram feces.

Anaerobic plates† inoculated with similar quantities of Suspension No. 2, Spores, were made on blood agar in 139 different stools of this series. The medium was prepared by adding about 1 c.c. of naturally sterile, defibrinated blood (of rabbit, dog, cat, or goat according to availability) to the tube of plain agar, melted and cooled to 45° C., immediately before pouring. The plates were incubated 48 hours under anaerobic conditions. An attempt was made to isolate as many different species as possible from these plates and to preserve them for further study. In the tables 26 types are indicated. Of these only six deserve serious consideration as the others are represented by a single occurrence only, except Type XV which

\* The work upon identification of the bacteria isolated from feces will appear in a subsequent communication.

† We owe this method to the suggestion of Professor Theobald Smith, who has found it well adapted to the isolation of *B. welchii* from feces.



occurred twice in the same individual. The types of sporogenic anaerobes which were repeatedly isolated were Numbers I, II, III, VIII, IX, and XVIII. Type I is undoubtedly the species described by Welch and Nuttall<sup>20</sup> under the name, *B. aerogenes capsulatus*. Type II resembles it very closely but forms spores more readily. Both of these hemolyze blood, producing clear areas around their colonies in the blood agar plates. Type III does not hemolyze the blood, but produces a green coloration about the colony on the blood agar plates. Type VIII appears to be related to *B. edematis* but is nonpathogenic to laboratory animals. In milk fermentation tubes it rapidly digests the casein without the formation of acid.\* Type IX resembles Type VIII very closely, being distinguished from it chiefly by a slower liquefaction of gelatin. Type XVIII is morphologically distinct from the others as it forms a large oval terminal spore much wider than the cell. In this it resembles Bienstock's<sup>2</sup> *B. putrificus*, the Köpfchen bacillus described by Escherich,<sup>5</sup> and the drum-stick bacillus described by Tavel.<sup>16</sup> It is quite probable that Types I and II are merely varieties of the same species, and that Types VIII and IX are also similarly related. If this should be the case there would be left for consideration only four species. In the examination of 139 different stools for sporogenic anaerobes according to this method, Type I was recognized in 113, Type II in 25, Type III in 21, Type VIII in 14, Type IX in 8, and Type XVIII in 12. The more detailed description of these forms must be left for a subsequent communication.

The number of colonies appearing upon these anaerobic spore plates was exceedingly variable. The greatest number, 64,398 cultivable anaerobic spores per milligram feces, occurred in Subject G, June 22, consisting almost wholly of Type I. In nine subjects, anaerobic spore organisms were found at every examination. Three individuals, Subject D, Feb. 27, Apr. 10, and July 1, Subject F, July 27, and Subject J, Mar. 30, May 15, and June 17, failed to yield cultures of sporogenic anaerobes by this method, upon the dates named, altho they were found at other examinations. The average of all the 139 examinations was 1,790 cultivable anaerobic spores per milligram fresh feces. It should be kept in mind that these figures represent spores, and not the total number of sporogenic anaerobes.

Table 9 summarizes the figures given in Table 8, together with the results of the microscopic count of the fecal bacteria of Table 2.

TABLE 9.  
BACTERIA PER MILLIGRAM FRESH FECES.  
SUMMARY OF COLONY COUNTS AND OF DIRECT MICROSCOPIC COUNTS.

Subject		Aerobic Agar	Anaerobic Agar	Aerobic Gelatin	Acid non-liq. P. ct.	Aerobic Spores	Anaero- bic Spores	Microscopic Count
A.....	Maximum	94,691	57,642	90,860	100.00	3,556	13,698	539,000,000
	Mean	35,632	31,583	35,421	99.30	411	3,595	394,000,000
	Minimum	7,100	10,000	6,400	97.00	4	97	244,000,000
B.....	Maximum	747,000	658,160	736,440	100.00	51	964	425,000,000
	Mean	133,856	108,443	127,415	99.04	20	155	301,000,000
	Minimum	14,960	9,840	9,160	92.00	7	6	151,000,000
C.....	Maximum	681,728	680,737	760,604	100.00	538	449	678,000,000
	Mean	103,645	112,947	105,927	98.21	100	83	461,000,000
	Minimum	1,787	4,160	7,780	83.00	6	4	341,000,000
D.....	Maximum	74,000	61,920	87,135	100.00	121	3,538	448,000,000
	Mean	26,637	24,099	27,950	99.67	23	485	305,000,000
	Minimum	2,420	2,900	3,140	98.00	3	0	103,000,000
E.....	Maximum	1,200,213	1,162,273	1,232,677	100.00	35	3,152	569,000,000
	Mean	279,210	268,963	280,917	99.92	18	422	424,000,000
	Minimum	30,600	36,000	41,500	99.00	3	13	252,000,000
F.....	Maximum	1,526,140	1,137,300	380,360	100.00	269	3,280	498,000,000
	Mean	176,164	153,134	125,947	99.86	50	555	403,000,000
	Minimum	13,380	12,640	12,040	99.00	6	0	263,000,000
G.....	Maximum	279,878	289,575	260,368	100.00	280	64,398	583,000,000
	Mean	101,097	93,231	121,363	98.57	48	6,401	400,000,000
	Minimum	1,360	6,440	7,040	90.00	2	204	179,000,000
H.....	Maximum	1,106,737	1,184,358	967,514	100.00	72	10,124	707,000,000
	Mean	231,697	172,035	272,905	99.83	25	1,922	475,000,000
	Minimum	13,700	7,830	49,520	98.00	4	15	322,000,000
I.....	Maximum	554,780	561,880	575,540	100.00	20	13,531	612,000,000
	Mean	162,333	145,784	223,921	99.87	12	1,466	402,000,000
	Minimum	7,650	10,300	17,440	99.40	4	73	251,000,000
J.....	Maximum	88,093	86,515	80,395	100.00	40	21	476,000,000
	Mean	32,117	27,501	38,108	99.11	13	7	328,000,000
	Minimum	2,320	3,240	5,600	96.00	0	0	158,000,000
K.....	Maximum	244,000	212,000	83,660	100.00	181	26,361	385,000,000
	Mean	48,913	49,619	39,347	98.48	36	2,877	287,000,000
	Minimum	3,860	5,000	4,220	95.00	2	14	198,000,000
L.....	Maximum	306,580	264,840	262,320	100.00	60	39,449	507,000,000
	Mean	63,568	58,896	81,742	99.53	25	3,514	371,000,000
	Minimum	3,240	5,760	7,520	98.00	9	16	175,000,000
All subjects	Maximum	1,526,140	1,184,358	1,232,677	100.00	3,556	64,398	707,000,000
	Mean	116,230	103,903	128,414	99.38	65	1,790	379,000,000
	Minimum	1,360	2,900	3,140	83.00	0	0	151,000,000

It is clearly evident that the number of bacteria brought to development by our culture methods is very small in comparison with the result of the direct counts, on the average about one germ in three thousand appearing as a colony upon the plates.

## RELATION BETWEEN CULTIVABLE AND VISIBLE FECAL BACTERIA.

That only a small fraction of the bacteria of the human feces appears in cultures has been well known for some time (see Schmidt und Strasburger,<sup>12</sup> pp. 281-86). Klein<sup>10</sup> considers the bacteria which fail to develop to be dead and their death to be the result of specific germicidal action of the intestine and its secretions. From examinations of the intestinal flora of the rabbit he concluded that the bacteria did not multiply at any part of the digestive tract, but were being killed at every point and being disintegrated, especially in the large intestine. Klein's deduction from his experiments meets a serious difficulty in the well known fact that enormous numbers of bacteria continue to be excreted in the feces even when excluded from the food. Belonowski<sup>1</sup> has fed mice sterilized food exclusively for seven months without any appreciable diminution in the excretion of cultivable fecal bacteria. Moreover, the germicidal action of the digestive juices, with the exception of the gastric juice, has not been demonstrated. In fact, the bile, the pancreatic juice, and the enteric secretion are all fairly good culture media *in vitro*. The first effect of a germicide upon bacteria is to inhibit growth and multiplication, and the enormous number of bacterial cells in the feces shows that the conditions for bacterial growth are exceptionally favorable in the intestinal tract, rather than inhibitive, not to mention germicidal.

We may better understand the biology of intestinal bacteria if we keep clearly in mind the nature of bacterial growth and death, in general, as it has been observed in the culture tube and upon dead matter. In common with other living cells, bacteria utilize food materials, thus tending to exhaust the available supply, and they produce and excrete various substances, some of which are very unfavorable to their own growth and existence. It may be said that, in general, living cells are able to endure deprivation of food for a much longer period than they can endure the presence of the waste products of their own metabolism. That the products of bacterial growth exert a pronounced germicidal effect upon their own species is already well established as a general principle.\* Hehewerth<sup>7</sup> has shown this to be the case for *B. coli*, in cultures of which the bacterial cells begin to perish even before multiplication has ceased. In agar

\* Gotschlich in Kolle und Wassermann,<sup>11</sup> *Handbuch*, 1, p. 116.

flask cultures grown at 37° C. for five days and still perfectly moist, we have found the number of living bacteria estimated by the plate method to be, in one experiment,  $\frac{1}{15}$  of the total cells counted microscopically; in another experiment  $\frac{1}{8}$  of the total visible cells. In agar flask cultures incubated eight days at 37° and still perfectly moist, only  $\frac{1}{40}$  of the cells counted microscopically were found capable of development on agar plates. Each one of these visible cells was at the moment of its origin essentially capable of independent proliferation, given favorable medium and environment. Its death necessitates the assumption of definite unfavorable influences present in the culture. This condition of a culture has long been spoken of as exhaustion of the medium, after the conception of Pasteur that the nutritive substances were entirely used up and the bacterial cells starved to death, but in reality the exhaustion of the medium is due in a much larger measure to the accumulation of waste products. This fact will be made clearly evident by a consideration of the natural limitation of bacterial growth in cultures.

Eijkman<sup>4</sup> has shown that when gelatin is inoculated with 6,000,000 living cells of *B. coli* per milligram of medium, no growth takes place, only gradual death and disintegration of the bacteria, and he has clearly demonstrated that bacterial growth in general is limited not by the exhaustion of the nutritive substances of the medium but by the accumulation of very definite *inhibitory* metabolic products of the bacteria, some of which at least are diffusible, thermolabile, and not appreciably filterable through porcelain. He was able to regenerate an "exhausted" medium by filtration through porcelain or by heating, in neither of which procedures is any nutritive substance added, and yet the resulting material again supported bacterial growth. He has also shown that these same inhibitory substances are present in human feces. These results have been confirmed by Conradi and Kurpjuweit,<sup>3</sup> who were able to detect this inhibitory substance in cultures of *B. coli* after the first hour of growth at 37° C., and in human feces even when diluted 10,000 times. They suggest the name "Autotoxine" for this class of substances. These experiments merely bring more clearly and more definitely before us what has been evident in a vague sort of a way to anyone who has made plates or transplanted stock cultures.

Besides this unfavorable effect upon their own species, bacterial products also frequently exert an inhibitory and germicidal action upon other species, and this hetero-antagonism has been especially studied in the case of *B. coli* by Eijkman,<sup>4</sup> and by Conradi and Kurpjuweit.<sup>3</sup> It seems evident, therefore, that Escherich<sup>5</sup> was correct in ascribing to the concentration of intestinal contents by absorption of water and the consequent accumulation of bacterial waste products, an important rôle in restricting, weakening, and destroying the intestinal bacteria, and it appears safe to assume that a large part of the great mass of fecal bacteria which fail to grow are actually dead cells, killed not by the action of the body cells, but largely by the same causes which kill bacteria in artificial cultures, their own metabolic products.

Some of those individual cells of readily cultivable species which fail to develop may not be dead but only weakened so that they do not grow upon solid media, altho they may develop in broth or other more favorable media. Where bacteria are dying there are some almost but not quite dead. This conception serves to account for the discrepancies between quantitative estimations of the same species on different media, as has been pointed out by Escherich.<sup>5</sup>

The remarkable variations in the number of cultivable *B. coli* in the feces of the same individual are best accounted for, not by differences in the amount of multiplication of this species in the intestine but by the differences in the extent to which the bacterial cells have died before passage of the feces. From the researches of Escherich<sup>5</sup> and of Tissier<sup>8</sup> upon nurslings, of Tissier<sup>9</sup> upon older children, and of Gessner<sup>6</sup> upon adults, it is evident that bacteria of the *B. coli* group (including *B. lactis aerogenes*, Escherich) are the chief bacteria of the small intestine and reach their highest development in the cecum and upper colon, thus belonging to the flora of the upper part of the intestine rather than the lower. This group would, therefore, be especially subject to unfavorable influences in the lower colon, specific antagonistic substances resulting from its own excessive proliferation at higher levels, the drying of the intestinal contents with consequent crowding of bacterial cells, the absorption and exhaustion of nutritive material, and possibly the antagonistic action of other bacterial species better adapted to the conditions of the



lower part of the intestine. This last mentioned factor has been shown to be of particular importance in breast fed infants by Tissier.<sup>18</sup>

In general the colon bacilli flourish where the digestion of proteins and sugars is in progress, in the presence of abundant intestinal juice, and they perish as absorption is being completed. Other things being equal, the survival of a large number of *B. coli* in the feces would indicate that these conditions favorable to it had persisted until the moment of excretion from the body was more nearly approached than is ordinarily the case, due either to increased rapidity of peristalsis in the large intestine, or to incomplete or delayed absorption of water, protein, and possibly of carbohydrate. Those stools which gave rise to less than 10,000 colonies of *B. coli* per milligram feces and those which gave rise to more than 200,000 colonies per milligram have been selected for comparison especially in regard to the clinical examinations of the feces. The clinical examinations of the feces were made for the most part by Dr. W. S. Chapin, clinical assistant in the laboratory. In the tables, (+) indicates a very small amount or trace; +, a small amount; ++, a moderate amount; +++, considerable; +++++, a large, and ++++++, a very large quantity. The examinations for striated muscle, starch, granulose bacteria, and fat were made with the aid of the microscope. Table 10 presents the data for the 29 stools with less than 10,000 bacterial colonies per milligram. None of these stools showed mucous flakes, and only three, Subject G, Jan. 2, Subject K, Jan. 16 and Mar. 19, showed any excess of striated muscle fibers. In all the others the low colony count is associated with a fairly complete utilization of the muscle element of the food. In two of the exceptional cases, Subject K, Stools No. B 117 and No. B 163, there was undoubtedly marked fermentation due to anaerobes as indicated by the odor, abundance of gas bubbles in the feces, the greater number of colonies developed under anaerobic conditions, and the predominance of positive bacilli in the fermentation tube sediments. In the other exception, Subject G, Jan. 2, Stool No. B 107, the evidence is not so conclusive, but the anaerobic plates yielded 19,000 colonies per milligram and the glucose fermentation tube became almost filled with gas, 9.7 cm.

TABLE 10.  
CLINICAL DATA UPON THE STOOLS SHOWING LESS THAN 10,000 CULTIVABLE *B. coli* PER MILLIGRAM FECES.

Subject	Lab. No.	Date	Colonies per mg.	Consistency	Dry Substance P. ct.	Odor	Gas Bubbles	Mucous Flakes	Striated Muscle	Starch	Granulose Bacteria	Fat
A.....	B 60	Nov. 26	7,100	Formed	....	Strong	o	o	+	+	+	+
	B 246	July 6	9,800	Formed	25.2	Normal	o	o	+	o	+	+
	B 39	Nov. 13	1,787	Formed	....	Normal	o	o	(+)	o	+	+
	B 62	Nov. 26	4,786	Formed	....	Normal	o	o	+	+	+	+
	B 154	Mar. 6	7,400	Mushy	20.1	.....	o	o	+	+	+	+
B.....	B 166	Mar. 23	3,160	Formed	30.4	Normal	o	o	+	+	+	+
	B 100	April 22	9,100	Formed	24.3	Very strong	o	o	+	+	+	+
	B 135	Dec. 23	8,907	Formed	....	Strong	+	+	+	+	+	+
	B 103	Feb. 10	7,620	Formed	20.5	Sweetish	+	+	+	+	+	+
	B 157	Mar. 11	6,680	Formed	25.0	Sweetish	+	+	+	+	+	+
C.....	B 103	Mar. 27	2,420	Mushy	20.2	Sweetish	+	+	+	+	+	+
	B 65	Nov. 29	1,800	Formed	....	Normal	o	o	+	+	+	+
	B 88	Dec. 14	1,360	Formed	....	Normal	o	o	+	+	+	+
	B 107	Jan. 2	9,100	Formed	....	Normal	o	o	+	+	+	+
	B 131	Feb. 3	5,120	Formed	30.5	Normal	o	o	+	+	+	+
D.....	B 164	Mar. 19	9,720	Formed	29.2	Normal	o	o	+	+	+	+
	B 204	May 11	7,830	Formed	31.1	Strong	o	o	+	+	+	+
	B 101	Dec. 26	7,650	Formed	....	Normal	o	o	+	+	+	+
	B 31	Nov. 9	2,900	Soft	....	Normal	o	o	+	+	+	+
	B 54	Nov. 21	2,720	Soft	....	Strong	o	o	+	+	+	+
E.....	B 100	Dec. 23	6,026	Formed	....	Sweetish	+	+	+	+	+	+
	B 207	May 15	2,320	Formed	22.4	Sweetish	+	+	+	+	+	+
	B 243	July 4	4,900	Mushy	21.4	Normal	+	+	+	+	+	+
	B 117	Jan. 16	8,370	Mushy	....	Offensive	+	+	+	+	+	+
	B 163	Mar. 19	3,860	Formed	18.7	Putrefactive	+	+	+	+	+	+
F.....	B 23	June 5	6,240	Formed	24.5	Normal	+	+	+	+	+	+
	B 74	Dec. 6	3,240	Soft	....	Normal	+	+	+	+	+	+
	B 97	Dec. 20	9,500	Formed	....	Normal	+	+	+	+	+	+
	B 155	Mar. 9	5,140	Formed	38.6	Strong	+	+	+	+	+	+
	B 155	Mar. 9	5,140	Formed	38.6	Strong	+	+	+	+	+	+

In Table 11, similar data are given for the 32 stools yielding more than 200,000 *B. coli* colonies per milligram. In 12 of these, mucous flakes were observed and the 3 counts above a million per milligram were all associated with the presence of excessive mucus. In 15 of the 32, striated muscle was present in increased quantity. In 6, the dry substance of the stool was less than 20 per cent. There are 6 of the 32 stools in which no excess of mucus, muscle residue, or water was noted. A comparison of these two tables suggests a definite relation between these signs of incomplete absorption, increased intestinal secretion, and accelerated peristalsis on the one hand, and a large number of cultivable *B. coli* in the feces on the other, but it is also clear that other factors enter into and confuse this relation. The extent of multiplication of *B. coli* in the intestine seems to have very little relation to variations in the number of cultivable *B. coli* in the feces, this latter depending upon factors which affect the extent of dying of the bacterial cells.

The assumption that all the visible bacteria which fail to develop on plate cultures are dead is, however, not justifiable. Special methods of culture may be expected to bring to development new species of fecal bacteria. Already several kinds of bacteria requiring special methods for their isolation have been shown to be fairly constant inhabitants of the intestine, of which the acidophile or acid resisting group and the sporogenic anaerobes may be mentioned. Besides these, there are certain forms such as the slender spirochetes which are visibly alive in the feces, and yet have not been brought to development by culture methods. It is not improbable that some species, cultivable with the greatest difficulty, may be very important members of the fecal flora, as their adaptation to such a strictly parasitic existence would suggest. To estimate the quantity of the living fecal bacteria not cultivable by our present methods, is manifestly impossible, and until we possess some better criterion by which to recognize living and dead bacteria, the results of culture experiments must be interpreted with caution. One must always keep in mind the possibility that some of the species under consideration may not be cultivable by the methods employed.

TABLE II.  
CLINICAL DATA UPON THE STOOLS SHOWING MORE THAN 200,000 CULTIVABLE *B. coli* PER MILLIGRAM FECES.

Subject	Lab. No.	Date	Colonies per mg.	Consistency	Dry Substance P. ct.	Odor	Gas Bubbles	Mucous Flakes	Striated Muscle	Starch	Granulose Bacteria	Fat
B.	B 56	Nov. 22	220,000	Mushy	.....	Strong	++	o	+	+	++	+
	B 79	Dec. 9	324,000	Mushy	.....	Strong	++	o	+	+	++	o
	B 150	Mar. 2	747,000	Mushy	19.0 (?)	Putrefactive	+++	++	+	(+)	++	+
C.	B 140	Feb. 20	359,924	Formed	17.3	Normal	o	+	+	+	+	+
	B 238	June 25	681,728	Formed	31.3	Strong	o	o	+	+	+	+
	B 262	July 27	223,840	Formed	23.3	Sweetish	o	+	+	+	+	+
E.	B 118	Jan. 16	231,000	Formed	.....	Normal	o	+	+	+	+	o
	B 142	Feb. 19	221,833	Formed	23.1	.....	o	+	+	+	+	o
	B 149	Mar. 2	1,200,213	Formed	27.3	.....	o	+	+	+	+	o
F.	B 161	Mar. 16	440,231	Soft	10.0	Normal	o	+	+	+	+	o
	B 185	April 16	209,920	Formed	28.6	Normal	o	+	+	+	+	+
	B 221	June 3	833,501	Mushy	23.2	Sweetish	o	(+)	+	+	+	(+)
G.	B 233	June 19	235,271	Formed	28.1	Putrefactive	+	o	+	+	+	+
	B 257	July 22	209,074	Formed	28.8	Strong	+	o	+	+	+	+
	B 225	June 8	1,520,140	Formed	28.6	Strong	o	+	+	+	+	+
H.	B 237	June 25	423,060	Formed	30.4	.....	o	+	+	+	+	+
	B 143	Feb. 21	228,358	Formed	29.9	.....	o	+	+	+	+	+
	B 212	May 20	279,878	Formed	29.9	Normal	+	+	+	+	+	+
I.	B 236	June 22	250,468	Mushy	19.5	Putrefactive	+	o	+	+	+	+
	B 248	July 9	275,179	Formed	31.2	Sweetish	+	o	+	+	+	+
	B 186	April 8	200,863	Formed	32.4	Strong	+	+	+	(+)	+	+
J.	B 216	May 25	222,495	Formed	27.0	Putrefactive	+	+	+	+	+	+
	B 240	June 29	551,304	Formed	23.9	Strong	+	+	+	+	+	+
	B 252	July 15	931,613	Part fluid	16.9	Putrefactive	+	+	+	+	+	+
K.	B 260	July 24	1,100,737	Formed	31.1	Putrefactive	+	+	+	+	+	+
	B 113	Jan. 12	222,800	Formed	.....	.....	+	+	+	+	+	+
	B 220	June 1	554,780	Formed	25.1	.....	+	+	+	+	+	+
L.	B 232	June 17	266,460	Formed	28.1	Strong	+	+	+	+	+	+
	B 244	July 3	204,585	Formed	27.2	Normal	+	+	+	+	+	+
	B 250	July 20	542,186	Formed	27.0	Strong	+	+	+	+	+	+
L.	B 40	Nov. 14	244,000	Soft	.....	Normal	+	+	+	+	+	+
	B 215	May 25	306,580	Soft	18.2	Strong	+	+	+	+	+	+

## FERMENTATION TUBES.

Fermentation tubes of sugar broth were inoculated with the mixed fecal flora of each stool examined. The medium was prepared from fresh meat, fermented with *B. coli* according to Smith's method. To the resulting nearly sugar-free broth, two per cent of the particular sugar was added, the medium filled into fermentation tubes (without foot) and sterilized in the autoclave. Pure dextrose, levulose, lactose, and saccharose were the sugars used. Each tube was inoculated with 0.25 c.c. of Suspension No. 1 (equivalent to 0.0025 gm. feces). The length of the closed arm was very nearly 12.5 cm. in each case. The quantity of gas produced has been recorded in linear centimeters, and the amount of this gas absorbed by alkali as percentage of the total gas.

The results of these fermentation tube inoculations are recorded in Table 12. The amount of gas produced showed considerable variation. Thus in the dextrose broth, Subject A, Jan. 6, the entire closed arm was filled with gas, while in Subject L, Mar. 9, only 0.9 cm. was produced. Of the twelve individuals, Subject A showed the largest average amount of gas in the dextrose broth, 7.3 cm.; Subject F, the smallest, 3.9 cm. The average for the 12 subjects was 5.2 cm. gas in the dextrose broth. Similar variations in the amount of gas produced also occurred in the other kinds of sugar broth, as will be seen upon examination of Table 12 or of the summary table. It is of interest to note that on the average the lactose broth excelled in the amount of gas produced, and this is also true of the average figures of every one of the 12 individuals (see Table 13).

The portion of the gas absorbable by alkali varied as a rule from  $\frac{1}{6}$  to  $\frac{2}{5}$  of the total, corresponding roughly to the gas formula of the *B. coli* group.

From the results of plate cultures one might expect a predominant growth of *B. coli* in these fermentation tubes. Microscopic examination of the sediments in the tubes after 24 hours' incubation, however, always revealed a considerable variety of bacteria, gram-positive and gram-negative bacilli and cocci being constantly observed. The predominant type of organism in the gram-stained sediments\*

\* Differences in the character of the sediments of the different sugar broth tubes inoculated from the same stool were frequently noted.



is indicated in the table. Gram-positive rods were predominant in six instances, in Subjects A, H, and K. In four of these, in Subjects A and K, the closed arm of the fermentation tube was nearly or quite filled with gas. In the other two, in Subject H, the tubes were about half full of gas. The gram-positive bacilli associated with this marked abundance of gas were probably *B. welchii*. On the other hand, there were several instances of abundant gas production in which gram-negative bacilli were predominant in the sediments. No branched forms were ever observed. The interpretation of results of this character presents great difficulties. It will be necessary to study more fully the character and action of each of the various species present in these sediments before a rational theory applicable to their associated action can be formulated. So far, one fact is certain—the growth in these fermentation tubes inoculated with 2.5 mg. of normal human feces is not merely a culture of *B. coli* but a number of species are here brought to development, which fail to appear on the agar plates.

One object in undertaking these fermentation tests was to test the clinical significance of variation in the amount and composition of the gas produced. The results show very marked variation in the amount of gas even in healthy men, and it is evident that the cause\* of these variations is not simple.

At present, work is in progress to isolate the various species from these fermentation tubes and determine their identity and action upon the sugars in pure and in mixt cultures. So far as we have been able to discover, the gas production is due to the activity of bacteria belonging either to the *B. welchii* group or the *B. coli* group. These bacteria were present in practically all the stools examined in sufficient quantity, as shown by plate examinations, so that each fermentation tube inoculated with 0.0025 gm. feces would contain

\* Herter, to whose work<sup>9</sup> we owe this application of the fermentation tube, apparently considers these variations to be an expression and a measure of the alterations in the gas forming function of the aerogenic species occurring in the intestine, altho his recent statements<sup>9</sup> are not quite clearly specific on this point: "Observations were made twice weekly on the gas production of the mixed fecal flora in dextrose-bouillon fermentation tubes in the hope of detecting any influence that might possibly be exerted by the sodium benzoate on the gas forming function of the intestinal bacteria" (Benzoate report, p. 579). "It is thus clear that large doses of sodium benzoate strongly tend to depress the ability of the fecal bacteria to form gas. The explanation of this fact is not at present clear. The depression in gas formation is certainly not due to the presence of sodium benzoate in the feces, since it was not possible to recover benzoic acid in amounts sufficient to cause such an effect. But it may be due to some action of the benzoate on the bacteria of the digestive tract at higher levels than the colon, or to an action on the digestive juices" (Benzoate report, p. 605).



### SUBJECT B.

B 33.....	Nov. 10	3.7	3.5	4.9	3.3	5.5	3.0	3.1	2.9	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	...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SUBJECT C.

[illegible]

TABLE 12—Continued.  
SUBJECT D.

LABORATORY No.	DATE	INOCULATED WITH MIXED FECAL FLORA, UNHEATED										INOCULATED WITH STORE MATERIAL, HEATED AT 80° C. FOR 15 MINUTES								
		Sugar Broth					Litmus Milk			Litmus Milk			Blood Broth							
		Dextrose		Levulose		Lactose		Saccharose		Predominant Bacteria in Gram-stained Sediments	Gas Cms.	Coag- ula- tion	Diges- tion	Reac- tion	Gas Cms.	Coag- ula- tion	Diges- tion	Reac- tion	Gas Cms.	Cap- sules
		Gas Cms.	CO <sub>2</sub> %	Gas Cms.	CO <sub>2</sub> %	Gas Cms.	CO <sub>2</sub> %	Gas Cms.	CO <sub>2</sub> %											
B 34.....	Nov. 11	2.4	33	2.2	31	4.0	37	2.9	31	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
B 57.....	Nov. 23	3.5	29	2.0	20	3.7	30	3.3	30	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
B 80.....	Dec. 10	2.0	24	2.5	20	4.8	27	2.9	24	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
B 90.....	Dec. 23	3.8	20	3.1	19	3.9	50	2.3	35	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
B 112.....	Jan. 9	3.3	21	4.0	20	4.6	28	5.7	33	.....	1.2	+	o	Acid	.....	.....	.....	.....	.....	.....
B 123.....	Jan. 25	.....	.....	5.0	30	5.4	27	5.0	30	.....	2.0	+	o	Acid	.....	.....	.....	.....	.....	.....
B 135.....	Feb. 10	3.1	16	0.6	34	5.6	32	5.8	34	Negative cocci	0.5	+	o	Acid	.....	.....	.....	.....	.....	.....
B 147.....	Feb. 27	3.8	31	1.1	18	7.3	28	2.9	24	Mixed	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
B 157.....	Mar. 11	4.0	17	3.6	27	6.0	23	1.2	16	Mixed	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
B 160.....	Mar. 27	5.7	36	0.0	36	6.6	40	4.7	36	Mixed	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
B 181.....	April 10	5.7	31	4.5	35	6.0	41	5.7	35	Positive cocci	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
B 193.....	April 27	5.2	34	5.5	38	8.3	33	5.3	40	Positive cocci	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
B 205.....	May 13	3.0	26	3.3	24	2.8	25	3.3	24	Positive cocci	1.0	+	o	Acid	.....	.....	.....	.....	.....	.....
B 217.....	May 28	4.0	27	3.2	28	5.5	27	4.8	31	Positive cocci	3.0	+	o	Acid	.....	.....	.....	.....	.....	.....
B 220.....	June 15	4.0	30	2.0	20	4.5	24	1.7	23	Negative rods	2.5	+	o	Acid	.....	.....	.....	.....	.....	.....
B 241.....	July 1	3.0	30	4.4	31	5.3	32	3.5	28	Mixed	3.5	+	o	Acid	.....	.....	.....	.....	.....	.....
B 253.....	July 17	4.2	33	4.0	25	6.2	33	5.5	34	Positive cocci	3.0	+	o	Acid	.....	.....	.....	.....	.....	.....
B 265.....	July 31	1.7	17	0.5	..	5.8	32	6.2	33	Mixed	3.0	+	o	Acid	.....	.....	.....	.....	.....	.....
Average.....		4.0	29	3.8	27	5.7	33	4.3	30											
Minimum.....		1.7	16	0.5	18	2.8	23	1.2	16											
Maximum.....		5.7	36	6.6	36	8.3	50	6.2	40											







SUBJECT H.

B	48	Nov. 18	3.0	27	4.5	36	5.8	36	4.6	33
B B	71.	Dec. 3	4.5	3.0	27	4.6	30	1.1	18	
B B	94	Dec. 19	3.9	28	3.1	25	6.9	36	1.3	23
B B	100	Jan. 6	8.0	30	4.3	30	7.7	29	5.2	30
B B	121	Jan. 22	6.4	30	5.1	29	5.3	21	9.9	33
B B	133	Feb. 6	7.4	33	6.1	37	5.1	34	5.2	34
B B	145	Feb. 24	5.0	30	..	..	8.2	25	7.1	28
B B	156	Mar. 10	6.4	32	3.5	..	5.0	30	7.1	23
B B	168	Mar. 25	6.0	46	4.8	38	3.7	1.5	33	Negative rods
B B	180	April 8	5.7	31	4.5	31	6.0	30	2.8	30
B B	192	April 25	6.0	38	6.7	37	7.4	36	6.2	37
B B	204	May 11	6.1	20	4.8	29	8.5	35	6.1	29
B B	216	May 25	5.6	33	5.6	30	5.4	31	7.0	38
B B	228	June 12	6.8	35	..	6.3	33	4.5	31	Negative rods
B B	240	June 20	6.1	30	5.8	34	8.5	35	8.3	35
B B	252	July 15	7.5	30	5.7	35	5.5	30	7.1	33
B B	260	July 24	6.9	33	6.2	32	7.2	32	6.3	33
Average	Minimum	..	6.0	31	4.0	32	6.6	32	5.4	31
Maximum	..	..	8.0	46	6.7	38	8.5	37	9.9	38

SUBJECT I.

B	32	55	78	101	113	125	137	160	182	184	198	228	242	256	Average	Minimum	Maximum
Nov. 11	1	4	21	1	3	15	4	1	20	3	4	38					
Nov. 27	..	..	4	0	20	4	7	34	3	0	23						
Dec. 5	8	0	18	4	33	5	7	35	3	7	27						
Dec. 20	5	0	2	4	25	4	8	33	3	9	38						
Jan. 12	0	2	20	5	0	30	10	0	30	5	33						
Jan. 27	0	2	5	0	30	4	0	33	4	0							
Feb. 13	0	2	7	5	0	24	5	33	34								
Feb. 14	4	2	4	0	20	5	6	32	30	0							
Mar. 1	4	2	0	2	7	32	30	30	30	0							
Mar. 31	4	2	0	2	8	20	7	3	35	0							
Apr. 1	4	23	4	7	3	8	3	42	6	2							
Apr. 15	6	23	4	31	6	0	20	3	4	32							
Apr. 20	5	6	32	4	5	31	6	0	20	3	4	32					
May 13	5	2	27	3	2	28	5	3	3	2	31						
May 15	5	2	26	4	8	31	10	0	33	5	8	36					
June 1	5	7	31	0	0	35	7	5	33	5	4	35					
June 17	5	7	31	0	0	32	8	6	33	5	0	32					
July 3	6	7	34	5	0	32	7	3	35	6	7	37					
July 13	6	2	38	5	6	33	7	1	35	6	7	37					
July 20																	
Average	4	8	26	4	0	26	6	6	32	4	5	32					
Minimum	1	4	10	1	3	15	4	1	20	3	0	23					
Maximum	8	0	38	6	0	35	10	0	42	6	7	40					



SUBJECT K.

B 40-	Nov. 14	1	18	1	3	23	2	6	30	4	1	34
B 40-	Nov. 14	1	18	1	3	23	2	6	30	4	1	34
B 40-	Nov. 27	4	30	4	2	20	8	1	38	4	2	30
B 86	Dec. 13	34	3	5	28	5	1	20	4	2	3	30
B 105	Dec. 30	10	4	26	8	3	2	7	4	3	3	30
B 117	Jan. 16	6	3	34	5	5	20	0	7	1	10	3
B 129	Jan. 31	8	5	31	8	2	35	7	5	3	3	30
B 141	Feb. 10	6	2	27	4	4	38	6	5	3	10	7
B 151	Mar. 4	2	6	34	2	0	7	6	7	6	6	36
B 163	Mar. 19	5	0	30	..	..	..	0	5	2	9	2
B 175	April 3	5	1	23	5	5	4	3	9	0	2	34
B 187	April 20	5	6	37	6	7	40	8	5	30	8	5
B 199	May 4	5	3	33	2	7	5	38	3	1	25	
B 211	May 20	9	5	28	6	32	9	4	35	7	0	35
B 223	June 5	10	2	29	0	2	35	8	20	7	2	31
B 235	June 23	11	2	33	5	5	30	10	31	8	4	34
B 247	July 9	4	3	32	4	4	31	6	3	38	0	35
B 263	July 20	4	31	0	4	16	0	6	3	0	0	36
Average		6	2	30	5	0	3	8	1	3	6	3
Minimum		1	1	18	0	6	16	2	25	2	7	25
Maximum		11	2	37	9	2	43	10	8	4	10	7

SUBJECT I.

[illegible]

some bacteria of both groups. The small gas production occurring in some instances cannot, therefore, be ascribed to the lack of aerogenic organisms, nor is the diminished gas production due to a loss of the gas producing function in these aerogenic species, for mere pasteurization of mixt fecal flora restores the gas producing power so that milk and blood broth fermentation tubes inoculated with it become entirely filled with gas. The explanation is to be sought rather in the antagonistic action of other members of the mixt fecal flora, which are able to develop in the sugar broth fermentation tubes and to inhibit or limit the proliferation of the gas formers. One important feature of the fermentation tube cultures is the variety

TABLE 13.  
SUMMARY OF GAS PRODUCTION IN SUGAR BROTH FERMENTATION TUBES.

		Sub- ject A	Sub- ject B	Sub- ject C	Sub- ject D	Sub- ject -E	Sub- ject F	Sub- ject G	Sub- ject H	Sub- ject I	Sub- ject J	Sub- ject K	Sub- ject L	All Sub- jects
Dex- trose	Maximum	12.8	6.8	7.3	5.7	0.6	7.4	10.0	8.0	8.0	8.2	11.2	8.6	12.8
	Mean	7.3	4.4	5.4	4.0	5.3	3.9	6.1	6.0	4.8	4.4	6.2	4.1	5.2
	Minimum	4.8	2.0	2.1	1.7	1.8	1.3	2.3	3.0	1.4	1.6	1.1	0.9	0.9
Levu- lose	Maximum	10.5	5.7	7.2	6.6	8.2	6.1	9.0	6.7	6.0	7.4	9.2	10.3	10.5
	Mean	5.5	4.1	4.8	3.8	4.6	4.0	4.7	4.9	4.0	4.3	5.0	3.9	4.5
	Minimum	2.5	1.3	1.7	0.5	1.7	1.4	0.8	3.0	1.3	1.3	0.6	0.4	0.4
Lac- tose	Maximum	13.3	9.3	9.1	8.3	9.0	7.6	10.7	8.5	10.9	7.6	10.8	9.0	13.3
	Mean	7.8	6.5	7.3	5.7	6.9	4.6	7.6	6.6	6.6	5.9	8.1	5.4	6.6
	Minimum	3.2	3.3	3.2	2.8	3.5	1.3	3.0	4.6	4.1	2.5	2.6	1.9	1.3
Saccha- rose	Maximum	11.4	11.5	11.2	6.2	8.2	6.4	11.8	9.9	6.7	11.6	10.7	9.0	11.8
	Mean	5.2	4.6	4.7	4.3	4.8	4.3	5.5	5.4	4.5	4.9	6.3	4.5	4.9
	Minimum	0.4	1.4	2.2	1.2	2.1	1.0	2.2	1.1	3.0	2.4	2.6	1.3	0.4

of species brought to development, where the agar and gelatin plates show only *B. coli*. This is due not only to the liquid nature of the medium but to the anaerobic condition of the closed arm and also possibly to the opportunity for more intimate symbiosis afforded to the mixt bacteria, resembling in a measure the conditions of growth in the upper part of the intestine. It would be, however, a serious error to assume that the fermentation tube furnishes the same conditions as the intestine or that it allows the development of all species of fecal bacteria, or favors equally the multiplication of those species which do grow in it. It is merely an empirical method of demonstrating the presence of some living bacterial species which might otherwise be missed, and of studying the associated action of the fecal bacteria upon the liquid media. The kind of fermentation set up,



and the relative numbers of the different types of bacteria found in the sediment may, when some reliable means of interpretation has been furnished, prove of some value as an index of the number and activity of these different species in the intestine.

In Table 12 are also given the rather fragmentary results of some special tests for the immediate recognition of *B. welchii*, our Type I, and for the Type VIII (and IX), which completely digests milk. Litmus milk fermentation tubes, inoculated with 0.25 c.c. of Suspension 1, became coagulated with an acid reaction in every instance. The gas production was usually less than half the closed arm, but there are several instances in which the closed arm was filled with gas, indicating a marked development of *B. welchii*. No digestion of the casein was observed in these tubes. Litmus milk fermentation tubes inoculated with 0.50 c.c. of Suspension No. 2, Spores, usually became coagulated with an acid reaction and the closed arm of the tube entirely filled with the gas, indicating the unobstructed action of the gas bacillus. In a few instances the casein was digested without acid production and without the formation of gas, indicating the presence of Type VIII (or IX), and in some tubes there was evidence of the associated action of two or more sporogenic species. A comparison of the behavior of these two sets of milk fermentation tubes, the one inoculated with the unheated mixt fecal flora and the other with the same material after it had been heated to 80° C. for 15 minutes, indicates the marked influence which the asporogenic intestinal bacteria exert upon the behavior of the sporogenic forms.

The broth fermentation tube to which has been added a bit of naturally sterile liver or other animal tissue, as recommended by Smith,<sup>13</sup> was found to be especially useful in the rapid detection of *B. welchii*. Instead of the solid tissue we have usually added naturally sterile defibrinated blood to the broth, which appears to be equally serviceable. These tubes inoculated with 0.50 c.c. of Suspension No. 2, Spores, usually became filled with gas within 24 hours at 37° C., and the capsulated bacilli could be readily demonstrated by suitable staining of the sediments. This is the easiest and simplest culture method for recognizing the presence of the gas bacillus spores in feces.

## CONCLUSIONS.

1. The number of fecal bacteria brought to development on artificial culture media is a minute fraction of the total fecal bacteria microscopically visible.

2. Upon aerobic lactose gelatin plates, upon aerobic lactose agar, and anaerobic glucose agar plates, bacteria of the *B. coli* group are the only members of the normal fecal flora brought to development in a satisfactory manner.

3. Sporogenic aerobic bacilli are normally present in the human feces, and one species has been repeatedly isolated from each of the 12 individuals.

4. Sporogenic anaerobic bacilli may be isolated from nearly every sample of human feces, by appropriate methods. *B. welchii* is a normal constituent of the fecal flora.

5. The quantity of vegetative cells of the sporogenic species has not been estimated. The total number of sporogenic bacilli in the feces is therefore probably much greater than indicated by the cultures of spore material.

6. Among the visible bacteria which fail to grow, a considerable number are undoubtedly dead cells belonging to cultivable species. The death of these cells is primarily due to the unfavorable products of their own growth, the effect of which is exaggerated by the concentration of intestinal contents, the absorption of food material, and the antagonistic action of other bacterial species.

7. The variation in the number of cultivable *B. coli* in the normal feces is largely dependent upon the extent to which this bacterial destruction has progressed.

8. There are a number of bacterial species in normal feces, not yet brought to satisfactory development on plate cultures.

9. The fermentation tube filled with various liquid media is exceedingly valuable in bringing to development various species of fecal bacteria.

10. The gas produced in sugar broth fermentation tubes, inoculated with the mixt bacteria contained in 0.0025 gm. feces, is fairly constant in composition, but exceedingly variable in quantity.

11. This variation in the amount of gas produced is not necessarily due to an alteration in the aerogenic function of the gas-forming

fecal bacteria, but is due rather to variations in the extent to which other species develop in the fermentation tubes and restrict the action of the gas formers.

In conclusion it is a pleasure again to acknowledge the encouragement of Professor H. S. Grindley, chief of the laboratory, and the assistance of Professor Theobald Smith, whose counsel has been of especial value to us in this part of the work.

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# THE PROPORTION OF GRANULAR AND BARRED FORMS OF BACILLUS DIPHThERIAE IN THROAT CULTURES.\*

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IN a preliminary communication<sup>1</sup> in May, 1908, R. G. Perkins reported the occurrence, in the throat cultures submitted to the Bacteriological Laboratory of the City of Cleveland, of a bacillus morphologically very much like certain forms of *B. diphtheriae*. Two forms of such diphtheria-like organisms have been met with, one somewhat larger than the other. Both are characterized by the possession of metachromatic polar granules. Because of the presence of these granules and of the size and shape of the bacilli there is a striking resemblance between these nonpathogenic forms and the types of true diphtheria bacilli labelled "C" and "D" in the exhaustive study upon the morphology of *B. diphtheriae* by Wesbrook, Wilson, and McDaniel.<sup>2</sup> At times there may be a central granule also, or only a single polar granule together with one more centrally situated. Such forms are much like Wesbrook's Type "C." In mixt cultures from the throat, incubated for from 12 to 24 hours, the presence of this organism has been very confusing. Frequently enough, longer incubation and even isolation in pure culture have been necessary definitely to determine whether the organism present in the culture was or was not the nonpathogenic form. With the attention directed to the possibility of the presence of the latter, a proper conclusion is usually reached, even in early cultures, by the fact that the polar granules of the nonpathogenic organism are of such a size as to give the body of the bacillus a cylindrical shape, a characteristic which is just as marked, however, in Wesbrook's Types "C" and "D," and by the absence of so marked a tendency toward parallel arrangement as occurs in *B. diphtheriae*.

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<sup>1</sup> Presented at the Eighth Annual Meeting of the American Association of Pathologists and Bacteriologists, Ann Arbor, Mich.

<sup>2</sup> *Eighteenth Report of the State Board of Health and Vital Statistics of Minnesota, 1899-1900*, p. 613.

That the liability of confusion and the possibility of mistakes in the bacteriological diagnosis of diphtheria might be lessened to a certain degree appeared probable from the following paragraph by F. P. Gorham:<sup>1</sup> "In the routine examinations for the diagnosis of diphtheria in our own laboratory, and Mr. Rickards informs us that the same is true in the Boston laboratory, the granular forms of the diphtheria bacillus have been exceptionally uncommon this fall. Their place is taken by the barred type, which have become now the most common form. Indeed there would hardly be a positive diagnosis at present were we to abide by the former rule to regard the granular types alone positive."

If the proportion of granular forms of the diphtheria bacillus in Cleveland cultures has decreased to such a degree as has occurred in the Providence and Boston cultures, then a diagnosis of diphtheria could be made from the presence of barred forms in the great majority of cases and the possibility of confusing the nonpathogenic organism with *B. diphtheriae* would be lessened in a corresponding degree. To determine this question of the relative frequency of granular and barred forms a record was kept of the prevailing types of the diphtheria bacillus occurring in the cultures submitted to the Bacteriological Laboratory of the City of Cleveland for the period of one year, from April 1, 1908, to March 31, 1909. A period covering one year, rather than one which would give a certain number of positive cultures, was chosen in order to determine whether there might be any relation between season and the frequency of the various types of bacilli found. Since the purpose of the statistics was to gain an idea of the relative frequency of granular and barred forms, solid forms were left out of consideration, and no attempt was made to classify the various types as completely as was done by Wesbrook and his coworkers. Altho no records of all the minor variations which were met with were kept, there were noted a few types of *B. diphtheriae* not described by Wesbrook. Whenever there was a possibility that the organism under consideration might be the nonpathogenic species pure sub-cultures were obtained. Of the morphological varieties not noted by Wesbrook, Wilson, and McDaniel, Type Ia approaches most nearly their Type "D," differing

<sup>1</sup> *Amer. Jour. Public Hygiene*, 1907, 17, p. 400.





from the latter chiefly in the more pointed ends and the smaller size of the polar granules. Bacilli of Type Ia have been grouped together with those of Type Ib under "pure granular" forms. Other types, which could be classified neither as pure granular nor pure barred forms, contain both granules and bars, are Types IIa, IIb, IIc. These are most like Wesbrook's Types "D," "E," and "F." They differ from the latter in the possession of polar granules and bear the same morphological relation to the latter types that Wesbrook's Type "G" bears to his Type "G<sup>2</sup>." Bacilli of Types IIa, IIb, and IIc have been grouped together as "granular-barred" forms, and have been counted under a separate head. If one wished to include them under either of the other two groups, granular or barred, they would more properly belong with the former, since the presence of granules is much more striking than the bars and is more helpful in making a diagnosis. These granular-barred forms may vary in size and in shape, but all are characterized by the possession of polar granules and barred protoplasm. A glance at Table 2 shows that they have been rather frequently encountered. In many of those cultures in which the granular-barred form was the predominating type a few pure granular and pure barred forms were also present. Under the barred types have been included those cultures containing bacilli with barred protoplasm but without granules, size and shape of the organism being left out of consideration, Type III. The bacilli included in

this group are most like Wesbrook's Types "C<sub>1</sub>," "D<sub>1</sub>," and "E<sub>1</sub>." The so-called involution forms have been included with the granular, barred, or granular-barred forms, depending upon the particular characteristics possessed.

Table 1 gives the total number of cultures examined during the year, together with the total number and percentage of positive

TABLE 1.  
TOTAL CULTURES EXAMINED.

Month	Total Cultures	Total Positive	Percent- age of Total Positive	Total 1st Cultures	Positive 1st Cultures	Percent- age of Positive 1st Cultures	Total Repeat Cultures	Positive Repeat Cultures	Percent- age of Positive Repeat Cultures
1908									
April.....	34	1	2.9	33	1	3.03	1	0	0.0
May.....	22	4	18.1	22	4	18.1	0	0	0.0
June.....	23	7	30.4	17	5	29.4	6	2	33.3
July.....	24	4	16.6	22	4	18.1	2	0	0.0
August.....	21	5	23.8	18	4	22.2	3	1	33.3
September.....	48	13	27.1	41	13	31.7	7	0	0.0
October.....	80	32	35.9	65	23	35.3	24	9	37.5
November.....	97	47	48.4	79	41	51.9	18	6	33.3
December.....	154	45	29.2	109	28	25.6	45	17	37.7
1909									
January.....	137	27	19.7	112	19	16.9	25	8	32.0
February.....	103	30	29.1	74	18	24.3	29	12	41.3
March.....	98	25	25.5	80	20	25.0	18	5	27.7
Totals.....	850	240	28.0	695	180	25.9	178	60	33.7

results, the number of first cultures, and the number of secondary cultures. The seasonal distribution of positive cultures is the usual one for Cleveland. At no time during the year was diphtheria epidemic.

Table 2 gives the total number of positive first cultures per month,

TABLE 2.  
POSITIVE 1ST CULTURES. PROPORTION OF VARIOUS TYPES.

Month	Total Positive	Granular	Barred	Both Granular and Barred	Granular-barred
1908					
April.....	1	1	0	0	0
May.....	4	3	1	0	0
June.....	5	2	0	3	0
July.....	4	3	1	0	0
August.....	4	3	0	1	0
September.....	13	3	0	3	7
October.....	23	11	1	7	4
November.....	41	21	1	9	10
December.....	28	18	0	1	9
1909					
January.....	19	11	1	1	6
February.....	18	15	0	0	3
March.....	20	12	0	0	8
Totals.....	180	103	5	25	47
Percentage of various types		57.2	2.7	13.8	26.1

classified according to the type of bacillus which predominated. Pure granular forms occurred with greatest frequency, cultures containing granular-barred forms were next most numerous, pure granular and pure barred types occurred in association in 25 cultures, and pure barred forms alone were met with only in five cases. Such a classification cannot, of course, be very exact. In placing the type encountered in a given culture under any of the four heads the predominating form alone was considered. It is quite certain that in the cultures included under any one group isolated bacilli of other types were also present.

TABLE 3.  
POSITIVE REPEAT CULTURES. PROPORTION OF VARIOUS TYPES.

Month	Total Positive	Granular	Barred	Both Granular and Barred	Granular-barred
1908					
April.....	0	0	0	0	0
May.....	0	0	0	0	0
June.....	2	1	0	1	0
July.....	0	0	0	0	0
August.....	1	0	0	1	0
September.....	0	0	0	0	0
October.....	9	4	0	1	4
November.....	6	3	0	1	2
December.....	17	12	0	0	5
1909					
January.....	8	4	0	0	4
February.....	12	8	0	1	3
March.....	5	3	0	1	1
Totals.....	60	35	0	6	19
Percentage of various types.....		58.3	0.0	10.0	31.6
Total positive cultures.....	240	138	5	31	66
Percentage of various types.....		57.5	2.08	12.9	27.5

In Table 3 are placed the positive secondary cultures. No pure barred cultures were met with among these. The remaining groups bear about the same relation to each other as in the first cultures. In both primary and secondary cultures the proportion of the various types to the total in each group remains approximately uniform.

It will appear at once that the results obtained in the Providence and Boston laboratories do not hold for cultures submitted to the Cleveland laboratory. Whereas, in the Boston and Providence cultures, barred types were so frequent that, according to Gorham, "there would hardly be a positive diagnosis at present were we to abide by the former rule to regard the granular types alone positive," in Cleveland barred forms have recently almost entirely disappeared.

Because of the predominance of granular forms we are still confronted with the possibility of confusing *B. diphtheriae* with the nonpathogenic organism described by Perkins. In connection with the small proportion of barred types in the Cleveland cultures it is interesting to note that the change in the prevailing type seems to be in a direction opposite to that noted in Boston and Providence. There, granular forms seem to have given way to barred types. Here, a comparison of the records of the past year with those of 1902 shows that barred forms have almost disappeared. In 1902, of 236 positive first cultures 94 (36.7 per cent) contained pure barred forms, 29 (11.3 per cent) contained barred and granular types in association, and 133 (51.9 per cent) contained granular forms alone.

What may be the causes underlying the production of a type containing both granules and bars, and what those which lead to changes in the prevailing type in different localities cannot be determined until the biological principles underlying the production of granules and bars in general shall have been better established.

#### SUMMARY.

Contrary to what has been noted elsewhere, the granular forms of *B. diphtheriae* in throat cultures sent to the Cleveland City Bacteriological Laboratory are increasing in frequency. Barred forms have almost entirely disappeared.

A type not present in earlier years, one not described by Wesbrook, Wilson, and McDaniel, is encountered with considerable frequency. Bacilli of this type contain both granules and bars. They vary in size and shape in cultures from different sources.

Granular types are present in over half of the positive cultures. Next in frequency are the granular-barred forms. In a fair proportion of cases both pure granular and pure barred forms are present in about equal numbers. Pure barred cultures are very infrequent. These proportions hold for secondary as well as for primary cultures.

The predominance of granular forms makes it necessary to bear in mind the occasional occurrence of a nonpathogenic bacillus which, in 12- to 24-hour cultures, is so much like certain of the granular types as to be confusing.

# BLEEDING TO DEATH IN ORDER TO OBTAIN MAXIMUM AMOUNT OF ANTIDIPHThERIC SERUM FROM HORSES.\*

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It is well known that horses, injected with diphtheria toxin for the production of antitoxin, show widely different reactions. It occurs occasionally that the reaction is so severe that the death of the animal is unavoidable. It is of importance in such cases to obtain as much blood from the horse as possible before death actually takes place.

During the past two years we have had six horses react so strongly to injections of the toxin that death was imminent. The first of these was bled from the jugular vein in the usual manner and a little over two gallons of blood were obtained. The next two horses were bled from the carotid and about four gallons were obtained. Glass

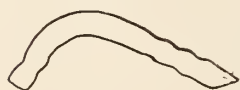


FIG. 1.

cannulae were used in these cases, but owing to the fall of the horses the cannulae were broken and much blood was lost. In order to avoid such accidents a structure was erected strong enough to support the weight of the horse after the latter had been suspended in a sling or the body held up by means of two stout ropes, one passing back of the forelegs, the other in front of the hind legs. The sling or the ropes are adjusted before operation is commenced and remain loose until the horse becomes weak from loss of blood. A cannula was then procured as shown in Fig. 1, about six inches long and nickel-plated. The inside measurement is  $\frac{3}{8}$  of an inch, the outside  $\frac{1}{2}$  an inch. The long arm, with beveled end, is inserted into the artery which has been ligatured above and held compressed by a hemostat below the incision. By such technic we obtained over five gallons of blood, the horse after a short struggle settling down into the sling.

It occurred to us that still more blood might be obtained by trans-

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fusion with salt solution. We have carried this out successfully with three horses and believe that the technic may be of use to others engaged in the production of diphtheria antitoxin.

The salt solution is placed in two 15-gallon jars, the jars being connected by a bent glass tube and supported by suitable means about four feet above the horse in order to obtain pressure by gravity. Another bent glass tube is then connected with a piece of rubber hose, the flow of the solution



FIG. 2.

being controlled by an ordinary pinch cock. An incision of five to six inches is made into the neck of the horse and a nickel-plated cannula, as shown in Fig. 2, about 6 inches long,  $\frac{1}{2}$  inch inside, and  $\frac{5}{8}$  of an inch outside, is inserted into the jugular vein, the upper part having been ligatured, the lower part being held by a hemostat. The rubber hose from the salt solution (the solution being approximately of blood temperature) is then carefully connected with the cannula and all air is excluded. When the vein is ready, the other cannula is similarly connected with the artery. A sterile rubber hose with a small piece of glass tubing is connected with the short end of the cannula, and when everything is ready the hemostat from the artery is removed and the blood collected. As containers we have used one-gallon bottles, previously sterilized, and covered with two layers of filter paper. These layers of filter paper are tied separately, so that one may be removed without disturbing the other. Previous to filling the bottles with blood, 50 c.c. of a 10 per cent solution of potassium oxalate are measured into each gallon bottle by means of a sterile 50 c.c. pipette.

The last horse which was bled to death in the manner described yielded 54 liters of blood. Twenty gallon bottles were used, and in order to determine how much of the resulting fluid was blood and how much salt solution, we took 10 c.c. from each of the 20 bottles and put these into 20 test tubes. These tubes were kept in an ice box for three days, and after the red corpuscles had settled, percentage measurements were made. The photograph shows the gradual falling of the amount of the red corpuscles. The first three gallon bottles were filled with blood before salt solution was admitted into the body of the horse, and in these the plasma is fully two-thirds. From

the fourth bottle the fall is gradual and fairly regular. Of the 54 liters of fluid obtained, 31 were blood, and of the 36 liters plasma obtained, 21 liters were actual plasma without salt solution. We

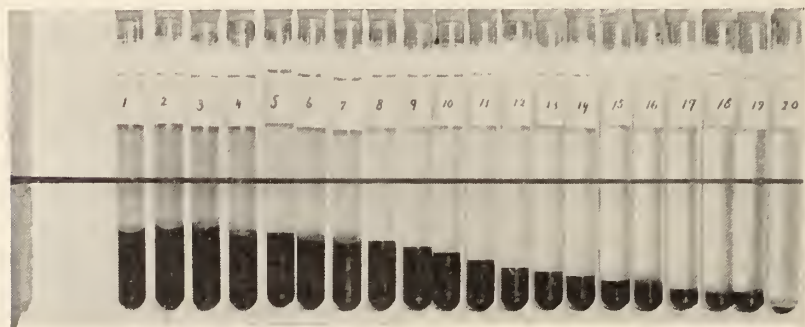


FIG. 3

consider this a high yield, since the horse was small, weighing less than 1,000 pounds.

By applying the technic described, we have obtained an amount of blood equal to the amount usually obtained by four bleedings. It is, therefore, profitable to bleed a horse to death in this manner if conditions are such as to warrant the procedure.

# ON THE USE OF ANHYDROUS SODIUM SULPHITE IN THE PREPARATION OF ENDO'S MEDIUM, TOGETHER WITH A NOTE ON THE PREPARATION OF ANHYDROUS SODIUM SULPHITE AND ITS STABILITY UNDER ORDINARY CONDITIONS.\*

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As is well known, Endo's medium is frequently employed as a means whereby to separate the typhoid organism from the colon bacillus, preliminary to further identification. According to Endo<sup>1</sup> this medium is prepared as follows:

Five hundred gm. ground beef, 10 gm. peptone, 5 gm. sodium chloride, and 30 gm. agar are added to one liter of water, the whole is well cooked, filtered, neutralized, and 10 c.c. of a 10 per cent soda solution added in order to make it alkaline, after which 10 gm. of c.p. milk sugar and 5 c.c. of an alcoholic solution of fuchsin are added as the result of which the medium becomes colored red. Then 25 c.c. of a 10 per cent solution of sodium sulphite are added, whereby the medium is gradually decolorized. It becomes entirely colorless, however, only after the agar has solidified. After the introduction of the medium into petri dishes it is sterilized for 30 minutes in a steam sterilizer. The author remarks first that the milk sugar must be chemically pure for the reason that the commercial product often contains cane sugar, from which the typhoid bacillus produces acids in consequence of which it becomes difficult to distinguish it from the colon bacillus; second, the sodium sulphite solution must be preserved in a well closed flask or should be prepared fresh for use; third, the alcoholic fuchsin solution must be previously filtered; fourth, the medium should be preserved in the dark, since as the result of the action of light it gradually acquires a red color. Petri plates prepared by the use of this medium are quite colorless and transparent. The colonies of the colon bacillus after 24 hours become deep red and show a greenish metallic sheen on the surface; on the other hand, the colonies of the typhoid bacillus are transparent and colorless, like minute drops of water.

Klinger<sup>2</sup> gives essentially the same directions for the preparation of this medium. Unfortunately neither of these authors gives any information regarding the particular form of sodium sulphite employed in the preparation of the medium. The results obtained by various workers in the Division of Pathology and Bacteriology of this labora-

\* Received for publication September 10, 1909.

<sup>1</sup> *Centralbl. f. Bakt., Orig.*, 1903, 4, 35, p. 100.

<sup>2</sup> *Arb. a. d. kais. Gesundheitsamtl.*, 1900, 24, p. 52.

tory point to the fact that the ordinary hydrated modification of sodium sulphite,  $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ , is the form ordinarily employed in the preparation of the medium and is the variety of the compound originally employed by Endo and also by Klinger. In this connection considerable difficulty has been experienced from time to time by the bacteriologists of the Hygienic Laboratory in obtaining a perfectly reliable and satisfactory Endo medium. For these failures two things seem to be primarily responsible: (1) impure lactose and (2) impure sodium sulphite. The first difficulty has been overcome by the use of Kahlbaum's c.p. lactose, and upon Kastle's suggestion the second difficulty has been overcome by the use of pure anhydrous sodium sulphite in half the quantity originally employed by Endo.

In this and other connections, considerable work has been done in this laboratory, during the past several years, on the sulphites, and a considerable number of samples of sodium sulphite, both of our own preparation and those obtained from chemical manufacturers, have been analyzed.

Thus a sample of heptahydrate of sodium sulphite ( $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ ) obtained from one of the most careful and reliable firms in the country gave the following results on analysis:

	Found (Per cent)
Sodium Sulphite . . . . .	22.05
Sodium Sulphate . . . . .	25.00
Water (loss on drying in vacuo at 100° C.). . . . .	52.50
	<hr/> 99.55

On the other hand, a fresh sample of the heptahydrate of sodium sulphite prepared in this laboratory gave the following numbers:

	Found (Per cent)	Theory for $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ (Per cent)
Sodium Sulphite . . . . .	47.58	49.99
Water . . . . .	52.11	50.01
	<hr/> 99.69	<hr/> 100.00

After standing for six months in a glass-stoppered bottle, under ordinary conditions, the bottle having been opened a few times during this interval in order to remove small amounts of the salt, the latter sample of sodium sulphite was found to contain only 40.84 per cent of sodium sulphite,  $\text{Na}_2\text{SO}_3$ . Hence under these conditions this sample of the

salt had lost approximately one-fifth of its total available sulphite. On the other hand, it has recently been pointed out by Hartley and Barrett<sup>1</sup> that anhydrous sodium sulphite,  $\text{Na}_2\text{SO}_3$ , is stable so long as it is kept dry, whereas they also have found that the hydrated salt,  $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ , oxidizes very readily in the air. And in this connection these authors have devised a method for the preparation and drying of anhydrous sodium sulphite out of contact with the air to which reference will be made in a subsequent part of this communication. It therefore occurred to one of us (Kastle) that in the preparation of the Endo medium much more regular and uniform results could be secured by the use of anhydrous sodium sulphite in half the quantities recommended by Endo and Klinger, the reason for taking half the quantity of sulphite being that the anhydrous salt contains almost exactly twice as much of the compound  $\text{Na}_2\text{SO}_3$  as ordinary sodium sulphite crystals, viz., the heptahydrate,  $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$  (see theory for  $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ , p. 620). The preparation of the Endo medium with anhydrous sodium sulphite and Kahlbaum's c.p. lactose was first carried out by Dr. Frost in the Division of Pathology and Bacteriology of this laboratory. One lot of the medium was prepared according to Endo's directions, using a 10 per cent solution of anhydrous sodium sulphite, and a second lot with a 5 per cent solution of the anhydrous sulphite. The typhoid and colon bacilli were found to grow upon both, the colon colonies becoming deep purplish red with a greenish metallic sheen, whereas the typhoid colonies were colorless. Dr. Frost found, however, that the growths of the two organisms were more typical when grown on the medium made up with the 5 per cent solution of anhydrous sodium sulphite. This also goes to show, as was suspected, that both Endo and Klinger used a 10 per cent solution of ordinary sodium sulphite crystals ( $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ ) in the preparation of the medium. In connection with the investigation of typhoid fever now being carried out in this laboratory large amounts of the Endo medium are used and in no instance has any difficulty been experienced in its preparation since these suggestions were made regarding the use of anhydrous sodium sulphite and chemically pure lactose for its preparation.

<sup>1</sup> *Jour. Chem. Soc., Trans.*, 1900, 95, pp. 1178-85.



The method now employed in the preparation of the medium is as follows:<sup>1</sup>

Ten gm. of Liebig's extract of beef, 10 gm. of peptone, and 5 gm. of sodium chloride are added to one liter of distilled water. This mixture is then heated until these substances have dissolved. It is then allowed to cool, and 40 gm. of powdered agar are placed on the surface. When this has settled, the mixture in a beaker is placed in an Arnold sterilizer, covered with paper, and allowed to cook for three hours. The solution is now made neutral to litmus paper with sodium carbonate. It is then filtered through cotton on a perforated funnel (Buchner filter), with the aid of the pump, or allowed to settle while slowly cooling, rejecting the turbid bottom portion. To the filtered solution 10 c.c. of sterile 10 per cent sodium carbonate solution are added. This medium may be conveniently preserved in quantities of 100, 200, and 400 c.c. in flasks, the flasks being considerably larger than is required for these quantities, in order to provide room for the other ingredients. In this way the medium can be stored until required for use, when the agar is melted and the other ingredients added as follows:

To each liter of the above medium add 10 gm. of c.p. lactose and 5 c.c. of freshly filtered alcoholic fuchsin, prepared by shaking 10 gm. of fuchsin (not acid fuchsin) with 100 c.c. of 96 per cent alcohol, allowing to stand 24 hours, decanting the supernatant fluid, and filtering this each time immediately before use. The medium is then vigorously shaken and placed unstoppered in the sterilizer for from five to ten minutes in order to allow the foam to settle, after which 25 c.c. of a freshly prepared, sterile, 5 per cent solution of anhydrous sodium sulphite are added. This is mixed into the medium by gently rotating the flask in order to avoid foaming. The Endo medium thus prepared is then sterilized for a few minutes in the Arnold sterilizer and poured into the petri dishes while it is steaming hot. After cooling, the medium should be nearly colorless to transmitted light, and rose or flesh colored to reflected light. The lactose, fuchsin, and sodium sulphite solutions must be added to the melted agar just before it is to be used. The plates are flown, and allowed to stand 20 minutes uncovered in the incubator in order to remove water of condensation and to obtain a good surface. Organisms which split lactose restore the red color of the fuchsin and appear as deep red, sharply defined, opaque colonies with a greenish metallic sheen; the typhoid organism produces smaller transparent colonies, resembling small drops of water.

The anhydrous sodium sulphite used by the bacteriologists of the Hygienic Laboratory in their recent work with the Endo medium was prepared by Elvove, using a modification of the method of Hartley and Barrett, which is described in the latter part of this communication, and which on analysis by the direct method of Giles and Shearer<sup>2</sup> was found to contain 99 per cent of anhydrous sodium sulphite,  $\text{Na}_2\text{SO}_3$ .

In this connection it seemed of interest to obtain some data as to

<sup>1</sup> See Report No. 3 on the "Origin and Prevalence of Typhoid Fever in the District of Columbia, 1909," *Bulletin No.*— of the Hygienic Laboratory, P. H. and M. H. S., pp. — (in press).

<sup>2</sup> *Jour. Soc. Chem. Ind.*, 3, p. 107; 4, p. 303.

the purity of a number of specimens of commercial anhydrous sodium sulphite, as well as additional data relative to the stability of commercial and other specimens of this salt under ordinary conditions. This has been done by Elvove.

Samples of anhydrous sodium sulphite were obtained from a number of chemical firms of recognized standing, including the well known firms of C. A. F. Kahlbaum, Merck and Co., and the J. T. Baker Chemical Co. As soon as these were received their purity was compared with the specimen of the salt which had been prepared by Elvove, after which they were kept for various lengths of time and analyzed at the end of each of the periods indicated, by the method of Giles and Shearer. The results of the analyses of the several samples are given in Table 1.

TABLE 1.  
COMPARISON OF THE PURITY OF SAMPLES OF ANHYDROUS SODIUM SULPHITE OBTAINED  
FROM VARIOUS SOURCES.

Number of Sample	Amount Taken for Analysis	N/10 Iodine Required	Percentage Purity
	gm.	c.c.	
1 (Commercial).....	0.1260	18.25	91.25
2 ".....	0.1260	18.75	93.75
3 ".....	0.1260	19.20	96.00
4 ".....	0.1260	19.30	96.50
5 ".....	0.1260	19.30	96.50
6 ".....	0.1260	19.45	97.25
7 (Prepared by Elvove's method).....	0.1260	19.80	99.00
8 (Prepared and analyzed by Hartley and Barrett, using an indirect method of analysis).....			99.87*

\* Another analysis by these authors showed the presence of 63.23 per cent  $\text{SO}_3$  or a purity of 99.62 per cent. However, on account of the indirect method of analysis (oxidation to sulphate and determining total sulphate) used by Hartley and Barrett, these figures do not necessarily show that their product actually contained this amount of anhydrous sodium sulphite,  $\text{Na}_2\text{SO}_3$ , and hence was purer than that obtained by the modified method which showed on analysis by the direct method of Giles and Shearer the actual presence of only 99 per cent of anhydrous sodium sulphite. Thus Hartley and Barrett found that 0.6674 gm. of their salt gave 1.2334 gm.  $\text{BaSO}_4$ . If this amount of their salt had actually contained a sulphate impurity of 0.62 per cent (the difference between the analyses compared) it would have altered the weight of the  $\text{BaSO}_4$  to the extent of only 0.0000 gm., i. e., only about 0.07 per cent. In other words, a sulphate impurity of 0.62 per cent would be concealed by as little as 0.07 per cent error in the gravimetric sulphate determination. On the other hand, by using the direct method of Giles and Shearer the impurity present is not minimized; and in a case as just mentioned would manifest itself by requiring about 0.65 c.c. of N/10 iodine less, which is an amount beyond experimental error. Further, the figures given for the percentage purity of the product obtained by the modified method simply show the purity of the sample which was used in this work and do not necessarily represent the highest purity obtainable by the modified method. As compared with the original method of Hartley and Barrett, there is also *a priori* no reason why the modified method should yield a salt of less purity, since all of Hartley and Barrett's precautions to avoid oxidation are included in the modified method; while there is reason why the latter might be expected to yield a product of even higher purity than the Hartley and Barrett method, since even the preliminary saturation with the  $\text{SO}_2$  is, in the modified method, conducted out of contact with the air.

These results show clearly that the specimen of anhydrous sodium

sulphite obtained by Elvove by a modification of Hartley and Barrett's

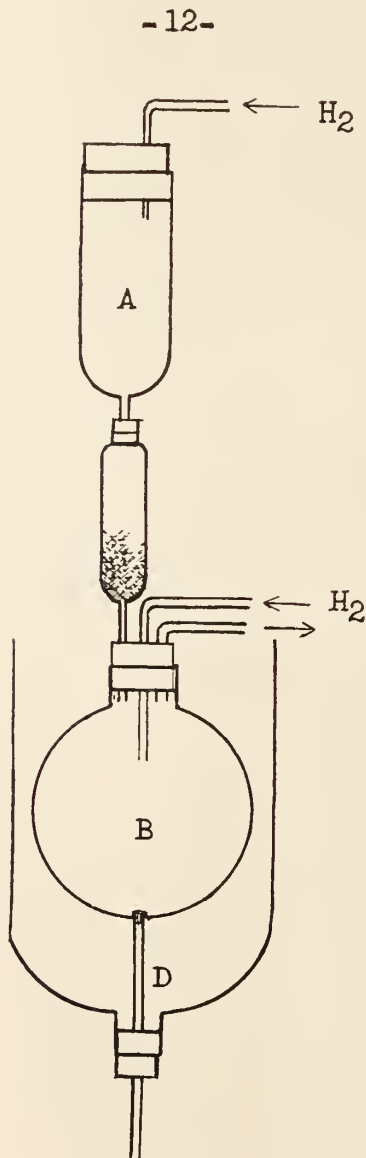


FIG. 1.—Diagram of Hartley and Barrett's apparatus.

being stopped by a small glass plug. The crystals were first washed with a mixture of alcohol and water, when with alcohol, and finally dried, all the operations being carried on in an atmosphere of hydrogen. The yield varied from 11 to 24 gm.

method is of greater purity than the best anhydrous sodium sulphite which we have thus far been able to obtain from chemical manufacturers. It is quite likely, however, that any of these lots of commercial anhydrous sodium sulphite are sufficiently pure for the preparation of a satisfactory and reliable Endo medium.

For the benefit of those who may wish to prepare pure anhydrous sodium sulphite, however, we will give a brief description of the method recently proposed by Hartley and Barrett for this purpose, and also the modification thereof employed by Elvove. Hartley and Barrett's method is as follows:

Forty gm. of Merck's pure sodium carbonate were dissolved in 120 gm. of air-free water, and a current of sulfur dioxide passed into the solution until the gain in weight showed that it was converted into sodium hydrogen sulphite; an equal quantity of sodium carbonate solution was then added, and the solution was quickly transferred to the vessel A (Fig. 1), containing hydrogen, whence it was filtered through glass wool into the vessel B, which was surrounded by a bath of brine kept at a temperature of over 100° by blowing in steam. As the anhydrous salt is less soluble than the hydrated form above 22°, it crystallizes when the temperature of the solution is raised and the yield is increased by evaporating the solution in a stream of hydrogen. After some hours, the solution was drawn off by the tube D, the crystals

While, therefore, this method gave an excellent product, it required a special apparatus and also a supply of superheated steam for keeping the brine bath at a temperature of above  $100^{\circ}\text{C}.$ ; the yield is small, the minimum being 11.6<sup>†</sup> per cent and the average only 18.4 per cent of what it should yield theoretically, and the time required

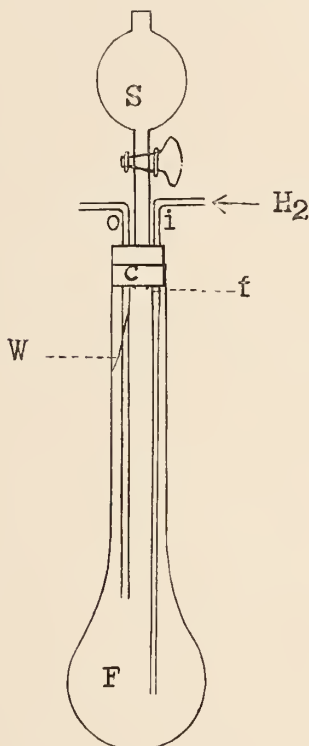


FIG. 2.—Modified arrangement.

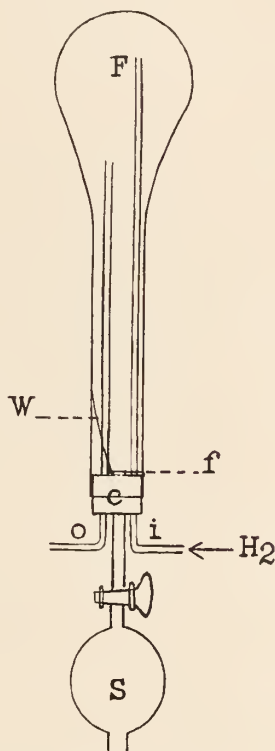


FIG. 3.—Modified arrangement (in position for draining off liquid).

is comparatively long. Inasmuch, however, as it appeared probable that the chief, if not the only reason, for effecting the evaporation by heating with steam instead of over a free flame is that the special apparatus required does not well adapt itself to the latter mode of heating, it therefore occurred to one of us (Elvove) that if we could

<sup>†</sup> These numbers are based on the assumption that the sodium carbonate referred to by Hartley and Barrett means the anhydrous sodium carbonate, since, as evaporation must be resorted to for concentrating the solution, they probably used as strong a solution as is feasible in the first place, making this solution from the anhydrous carbonate, or at least the monohydrate. Even had they used a hydrate of sodium carbonate, their yield would still have been smaller than that obtained in much less time by the modified method here described.

devise an arrangement which would permit of all the operations being conducted in an atmosphere of hydrogen and which could also conveniently be heated over a free flame, the necessity for a supply of superheated steam could be avoided and probably also one could obtain an increased yield in a shorter time. As a result, Elvove devised the following simple arrangement, which is illustrated diagrammatically in Figs. 2 and 3, and which was found to answer this purpose very satisfactorily.

*F* is an ordinary Kjeldahl flask, *S* is an ordinary globular separatory funnel. These are connected together by means of a well fitting cork, *c*, through which pass the hydrogen inlet tube, *i*, and outlet tube, *o*. The tubular end of the separatory funnel just passes through the cork, its end being nearly flush with the inner end surface of the cork, and has over it a muslin filter,<sup>1</sup> *f*. *W* is a piece of platinum wire one end of which is attached to the tubular end of the separatory funnel by insertion between the latter and the surrounding cork, while the other end touches the side of the flask, and which serves to lead the liquid from the separatory funnel into the flask and thus avoid the tendency of the liquid to flow partly down the outlet tube and escape. The connection of the apparatus to the hydrogen supply is made by means of soft rubber tubing (not shown in the figures) of sufficient length as to permit of the whole apparatus being inverted or moved about.

#### MODIFIED METHOD FOR PREPARING ANHYDROUS SODIUM SULPHITE.

Eighty gm. of pure anhydrous sodium carbonate were dissolved in freshly boiled water, using sufficient of the latter to make the total volume 240 c.c. Half of this was then transferred to the flask, *F* (Fig. 2), and sulfur dioxide passed into it to saturation, leading the exit gases into a tube containing a dilute aqueous solution of crystal violet which, in addition to serving as a liquid seal in preventing the entrance of atmospheric air into the flask, aids also in determining when the solution in the flask has become saturated with the sulfur dioxide. When the disappearance of the color in the crystal violet solution, as well as the odor of the exit gases, indicated that the liquid in the flask had become saturated with the sulfur dioxide, the supply of the latter was shut off and the inlet tube of the apparatus connected with the hydrogen supply, the inlet tube being raised above the level of the liquid in the flask, which now presented a perfectly clear solution (the crystalline precipitate which formed at first having been completely redissolved on further addition of the  $\text{SO}_2$ ), and the hydrogen allowed to pass through. The solution was then warmed for a few minutes, when the remaining half of the sodium carbonate solution was added through the separatory funnel,<sup>2</sup> *S* (Fig. 2). It was then boiled over a free flame until it was reduced in bulk to about one-third of the original volume, this reduction in volume being

<sup>1</sup> A convenient way of attaching the muslin filter consists in selecting a thin piece of soft muslin and cutting from it a narrow strip whose length is a little more than double the length of the cork and pressing its central part, by means of a suitable rod, completely into the opening in the cork which is intended for the tubular end of the separatory funnel and then gently work the latter in place.

<sup>2</sup> In using the separatory funnel as an inlet, the passage of the liquid may be hastened by connecting with a piece of rubber tubing and applying some pressure.



effected in less than one hour. By gently inverting the whole apparatus while still hot the crystals of  $\text{Na}_2\text{SO}_3$  were separated from the mother liquor, running the latter out through the muslin filter, *f* (Fig. 2). The crystals thus obtained were then washed twice with a mixture of equal volumes of water and alcohol, using 40 c.c. of the mixture each time; followed by three washings with absolute alcohol, using 20 c.c. of the latter for each washing. The whole apparatus up to the mouth of the flask was then immersed in an air oven<sup>1</sup> and heated to 110–120° C. for one hour, during which time the passage of the current of hydrogen remained uninterrupted. In this way 70 gm. of anhydrous sodium sulphite, or about 73.6 per cent of the theory, were obtained. An analysis by the direct method of Giles and Shearer showed the product thus obtained to contain 99 per cent of  $\text{Na}_2\text{SO}_3$ .

The chief advantages of this method over the original method of Hartley and Barrett may be summed up as follows:

1. No special apparatus is required, and no supply of steam is necessary.
2. The whole operation, including the saturation of the sodium carbonate with sulfur dioxide and subsequent drying of the salt, can be carried out in one apparatus.
3. The time required for evaporation is reduced to less than one hour.
4. The yield is increased from 11.6 to 25.3 per cent, to over 73.0 per cent.

#### THE STABILITY OF ANHYDROUS SODIUM SULPHITE UNDER ORDINARY CONDITIONS.

In order to obtain additional data relative to the stability of anhydrous sodium sulphite under ordinary conditions, a number of commercial samples of this salt, as well as that prepared by the method above described, were placed in paraffin-sealed bottles and also in ordinary glass-stoppered bottles which were kept without any special precautions on a table in the laboratory, and which during the time that samples were being drawn for analysis remained entirely exposed to the atmosphere of the laboratory. The results obtained are given in Tables 2 and 3.

As may be seen from the results given in Tables 2 and 3, anhydrous sodium sulphite is quite stable even when kept in glass-stoppered

<sup>1</sup> A convenient air oven for this purpose, in the absence of another better suited for it, may be made by cutting off a piece of sheet iron stove pipe to the proper length and placing a piece of sheet metal under the lower opening to serve as a bottom, while a similar piece of sheet metal, provided with a central opening to fit the neck of the flask and to allow the insertion of a thermometer and divided diametrically into halves across the opening, may serve as a top covering.

TABLE 2.

STABILITY OF ANHYDROUS SODIUM SULPHITE KEPT IN PARAFFIN-SEALED BOTTLES.

Number of Sample	Length of Time Kept	Amount Taken for Analysis	N/10 Iodine Required	Percentage Purity
	days	gm.	c.c.	
1.....	1	0.1260	18.25	91.25
1.....	15	0.1260	18.25	91.25
1.....	30	0.1260	18.20	91.00
2.....	1	0.1260	18.75	93.75
2.....	15	0.1260	18.70	93.50
2.....	30	0.1260	18.75	93.75
3.....	1	0.1260	19.20	96.00
3.....	15	0.1260	19.05	95.25
3.....	30	0.1260	18.95	94.75
4.....	1	0.1260	19.30	96.50
4.....	15	0.1260	19.30	96.50
4.....	30	0.1260	19.20	96.00
5.....	1	0.1260	19.30	96.50
5.....	15	0.1260	19.30	96.50
5.....	30	0.1260	19.25	96.25
6.....	1	0.1260	19.45	97.25
6.....	15	0.1260	19.30	96.00
6.....	30	0.1260	19.30	96.00
7.....	1	0.1260	19.80	99.00
7.....	15	0.1260	19.70	98.50
7.....	30	0.1260	19.70	98.50

TABLE 3.

STABILITY OF ANHYDROUS SODIUM SULPHITE KEPT IN GLASS-STOPPERED BOTTLES.

Number of Sample	Length of Time Kept	Amount Taken for Analysis	N/10 Iodine Required	Percentage Purity
	days	gm.	c.c.	
1.....	1	0.1260	18.25	91.25
1.....	3	0.1260	18.20	91.00
1.....	7	0.1260	18.25	91.25
1.....	15	0.1260	18.25	91.25
1.....	28	0.1260	18.20	91.00
2.....	1	0.1260	18.70	93.50
2.....	3	0.1260	18.70	93.50
2.....	7	0.1260	18.75	93.75
2.....	15	0.1260	18.70	93.50
2.....	28	0.1260	18.75	93.75
3.....	1	0.1260	19.10	95.50
3.....	3	0.1260	18.95	94.75
3.....	7	0.1260	18.90	94.50
3.....	15	0.1260	18.90	94.50
3.....	28	0.1260	18.05	94.75
4.....	1	0.1260	19.10	95.50
4.....	3	0.1260	19.10	95.50
4.....	7	0.1260	19.00	95.00
4.....	15	0.1260	19.00	95.00
4.....	28	0.1260	19.05	95.25
5.....	1	0.1260	19.30	96.50
5.....	3	0.1260	19.25	96.25
5.....	7	0.1260	19.20	96.00
5.....	15	0.1260	19.20	96.00
5.....	28	0.1260	19.25	96.25
6.....	1	0.1260	19.20	96.00
6.....	3	0.1260	19.25	96.25
6.....	7	0.1260	19.15	95.75
6.....	15	0.1260	19.20	96.00
6.....	28	0.1260	19.20	96.00
7.....	1	0.1260	19.80	99.00
7.....	3	0.1260	19.80	99.00
7.....	7	0.1260	19.75	98.75
7.....	15	0.1260	19.70	98.50
7.....	28	0.1260	19.75	98.75

bottles under ordinary laboratory conditions, thus confirming the experience of Hartley and Barrett<sup>1</sup> in this respect, who found that anhydrous sodium sulphite is stable so long as it is kept dry.

#### SUMMARY.

On account of its greater purity and stability under ordinary conditions, anhydrous sodium sulphite may be employed to advantage in the preparation of Endo's medium.

An improved method (Elvove's) for the preparation of pure anhydrous sodium sulphite is described.

Anhydrous sodium sulphite has been found to be quite stable under ordinary conditions, especially when kept dry.

A number of chemical manufacturers supply anhydrous sodium sulphite of sufficient purity for the preparation of a satisfactory Endo medium.

<sup>1</sup> *Loc. cit.*, p. 1179.

# I. EXPERIMENTS ON VACCINATION AGAINST RAT LEPROSY.\*†

## II. ON THE EXTRACTION OF RAT LEPROBACILLI FROM WATERY EMULSIONS BY MEANS OF CHLOROFORM.

### III. RAT LEPROBACILLI IN THE RAT LOUSE.

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#### I.

#### EXPERIMENTS ON VACCINATION AGAINST RAT LEPROSY.

TECHNIC of preparing the vaccine:

Vaccine I.—Pieces of the subcutaneous and glandular tissues from advanced cases of natural rat leprosy were ground up with powdered glass, extracted with 0.85 per cent sodium chloride solution, and the supernatant fluid pippered off into a tall cylinder. This was then heated in flowing steam for 30 minutes; when the coagulated albuminins had precipitated, the supernatant, opalescent fluid, rich in leprobacilli, was removed and autoclaved at 10 lbs. pressure for 30 minutes. When cool it was preserved with 0.5 per cent carbolic acid. An attempt was made to standardize the emulsion by counting the number of leprobacilli in  $\frac{1}{100}$  c.c. of a 1:100 dilution of the uncarbolicized emulsion. This showed that vaccine I contained more or less than 20,000,000 bacilli per c.c.

Vaccine II was prepared in the same way, excepting that it was simply heated once for 15 minutes at 20 lbs. pressure. It contained approximately the same number of bacteria per c.c.

1. EXPERIMENTS TO DETERMINE WHETHER VACCINATION WITH DEAD LEPROBACILLI WOULD INFLUENCE THE COURSE OF INOCULATION LEPROSY IN THE RAT.—White rats weighing 100 gm. were inoculated subcutaneously (abdomen) with an emulsion of rat lepro-

\* Received for publication August 18, 1909.

† Castellani attempted the vaccination of human lepers with emulsions prepared from their own tissues but I do not know where the results have been recorded. A similar independent attempt was made by P. G. Woolley in Siam. He, however, was unable to continue the treatment for a sufficient period. (*Proc. Soc. Exper. Biol. and Med.*, 1907, 4. p. 121.)

bacilli on February 20, 1909. This was the third passage through the white rat of a strain obtained from *Mus norvegicus* on February 13, 1908. Ten of these were vaccinated as follows and ten kept as controls. The vaccine was injected subcutaneously to one side or other of median line—the site of infection.

a) Nos. 7 and 8.—Treatment commenced two days after infection, with 5,000,000 dead lepra bacilli. Other injections were as follows:

TABLE 1. (VACCINE I.)		
Number of Injection	Number of Dead Lepra Bacilli Injected	Number of Days Since Last Injection
1	5,000,000	..
2	5,000,000	10
3	5,000,000	14
4	10,000,000	14
5	10,000,000	14
6	20,000,000	18
7	20,000,000	10

b) Nos. 9 and 10.—Treatment commenced 12 days after infection and received injections numbered 2-7 (Table 1).

c) Nos. 11 and 12.—Treatment commenced 26 days after infection and received injections numbered 3-7 (Table 1).

d) Nos. 13 and 14.—Treatment commenced 38 days after infection and received injections numbered 4-7 (Table 1).

e) Vaccine I had been kept for eight months before it could be used, so Nos. 15 and 16 received injections corresponding to numbers 5, 6, and 7 (Table 1) of vaccine II which was about one month old.

RESULTS.—Rat 9 died four days after the last injection and was found to be infected, and likewise with No. 11 which died nine days after the last injection. The remaining eight vaccinated rats were chloroformed 78 days after the last injection along with an equal number of infected controls. There was practically no difference in the extent to which the disease had progressed in the treated and untreated rats, the area of infection being about 4 cm. in diameter and extending to the inguinal glands.

2. EXPERIMENTS TO DETERMINE WHETHER VACCINATION WOULD PRODUCE ANY IMMUNITY TO SUBSEQUENT INOCULATION.—A large adult *Mus norvegicus* (M.n. 1) ( $250 \pm$  gm.) and a white rat (25) ( $100 \pm$  gm.) were vaccinated with 15 and 10 million bacilli in two separate injections, 14 days apart, of 10 and 5 million and 5 and 5 million bacilli respectively.

Seven days after the last injection they, along with two controls



of about the same weight (M.n. 3), and white rat 27, were inoculated with 1 c.c. of a broth emulsion of living rat lepra bacilli. They were chloroformed 127 days after the injection of the living lepra bacilli. M.n. 1 showed a slight amount of infiltration at the site of inoculation covering an area of 15 by 5 mm. The inguinal glands were not enlarged. Microscopically lepra bacilli were numerous at the site of inoculation but none were found in smears from the inguinal glands.

White rat 25 showed no signs of infection excepting three scattered whitish nodules, 1-3 mm. in diameter, in the fascia of the abdominal muscles at the site of the inoculation. The 3 mm. nodule contained caseous pus which microscopically showed numerous lepra bacilli. No bacilli could be found in the inguinal glands.

Control M.n. (3) showed an area of leprous infiltration about two inches long by one inch wide at the site of inoculation and its inguinal glands were considerably enlarged. Lepra bacilli were very numerous at the site of inoculation and in the inguinal glands.

Control white rat 27 showed an area of leprous infiltration about three inches by 1.5 inches at the site of inoculation and its inguinal glands were considerably enlarged. Smears showed numerous bacilli in the glands and at the site of inoculation.

Other rats in this series are still under observation.

SUMMARY.—Injection of an emulsion of rat lepra bacilli, killed in the autoclave, in doses varying from 5 to 20 million bacilli at intervals of 10 to 14 days, failed to arrest the progress of inoculation leprosy in the white rat even when treatment was commenced 48 hours after infection.

One *Mus norvegicus* and one white rat received two injections of the vaccine, on the 21st and 7th day respectively, before inoculation with living lepra bacilli. Here the progress of the disease was markedly delayed as compared with its progress in two controls.

## II.

### ON THE EXTRACTION OF RAT LEPPA BACILLI FROM WATERY EMULSIONS BY MEANS OF CHLOROFORM.

The subcutaneous tissue and glands from a leper rat were ground up with powdered glass and extracted with 0.85 per cent sodium

chloride solution. This clouded emulsion was shaken up with commercial chloroform which took on a clouded appearance. When drops of the chloroform were evaporated and the residue stained it was seen that millions of lepra bacilli had been extracted free from all cellular elements and other bacteria. The possible value of this fact in aiding the diagnosis of human leprosy has not yet been determined. Possibly it might help one in detecting lepra bacilli in the nasal secretions of an early case.

### III.

#### RAT LEPROSY BACILLI IN THE RAT LOUSE.

A leper rat (*M. norvegicus*, adult male) in a very advanced stage of the disease was seen to be literally covered with louse eggs. Only a few lice could be found. Six of them (*Haematopinus spinulosus*) were ground up on a slide and stained for lepra bacilli. Several hundred acid proof bacilli resembling the bacillus of rat leprosy were found scattered about in what appeared to be the granular contents of the intestinal tract.

These examinations were made in April, 1909, and are the only ones made on rat lice taken from leper rats since the publication of a previous note.<sup>1</sup> A number of lice from normal rats were examined for acid proof bacilli with negative results.

<sup>1</sup> *Jour. Infect. Dis.*, 1908, 5, p. 500.

## THE EFFECT ON MICE OF MINUTE DOSES OF B. ANTHRACIS.\*

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ONE occasionally meets with the statement that minute accidental inoculations with various microorganisms in amounts too small to cause a noticeable reaction may immunize or, possibly, hypersensitize to further inoculations. The object of the experiments given below has been to investigate this matter as regards mice and anthrax, making use of doses ranging from one spore or one bacillus upward; and, further, to obtain some data as regards infection, especially regarding the minimum lethal dose of this organism for mice under different conditions. Mice and anthrax were chosen because of the extreme susceptibility of the animal and the possibility of manipulating the microorganism in a virulent condition both in the vegetative and the spore form. This combination of extreme susceptibility and extreme virulence gives, perhaps, the most severe test in an investigation on immunity. Further, as regards infection, anthrax is an organism to which man and the lower animals are many times exposed in small subcutaneous doses, the majority of which, doubtless, never lead to infection; and it might be of interest to ascertain the effect, if any, of such doses on a susceptible species.

The same race of anthrax was used throughout the whole series and various dosage employed. The same dose was sometimes repeated over a considerable interval, and sometimes gradually increased or decreased in amount. For the most part virulent material taken directly from an animal was employed, though some series were carried out with spores or vegetative material grown in culture. It may be stated at the outset that results as regards the attainment of any degree of immunity or hypersensitiveness were negative.

The size of the dose was estimated under the microscope. Spores were counted, and each filament of the vegetative form measured with the eyepiece micrometer, except in relatively few cases, desig-

\* Received for publication July 24, 1900.

nated below, where larger doses were estimated less closely. In the isolation and inoculation of material a special technic involving the use of capillary pipettes was employed. This technic has been described in previous papers,<sup>1</sup> and only the special part of it developed in connection with this paper, the inoculation technic, need be described here.

Droplets containing single elements, or small groups of elements, were first isolated. Then with a fresh pipette containing a quantity of sterile physiological salt solution, the required dose was taken up. Before removing the pipette from the apparatus its tip was brought into contact with a large hanging drop of sterile salt solution suspended under the cover, and enough liquid admitted to wash the bacteria some distance back from the tip of the pipette. This is done to prevent the loss of the dose by the breaking off of the more delicate part of the tip in inoculating. A small portion of this tip may be purposely broken off before inoculating, since both the penetration of the skin and the discharge of the liquid are thus made easier. When the tip is inserted into the subcutaneous tissues of the animal, the contents are forced in by blowing into the rubber tube attached to the pipette. The liquid already in the pipette before taking up the dosage material serves to wash the bacteria out of the pipette. A new pipette is made for each inoculation.

For making inoculation pipettes a tough glass and tubing of somewhat thicker wall than is necessary for ordinary pipettes are used, and the portion just above the capillary tip is made somewhat thicker. The pipette is held in the right hand between the first finger and the thumb, and the second finger is extended to the bend in the pipette in order to press the tip through the skin. The mice were held in a short cylindrical holder, the tail brought through a small opening in a cloth placed over the open end of the holder, and, together with the edges of the cloth, held firmly to the side of the cylinder by means of a strong rubber band. The mouse could then be held in any desired position, the curve of the tail near the body making a convenient holding place for the left hand, leaving the right free for manipulating the pipette. Inoculations were made under low magnification furnished by large hand-lens supported on a jointed stand.

It is, of course, vital to the success of this technic that the bacteria pass into the animal with the inoculating fluid and none remain behind in the pipette. As a control a series of experiments was carried out in which the procedure was the same as in the mouse inoculations, except that the pipettes were discharged into suitable culture medium instead of into the animal. In one series 15 anthrax threads from cultures, five being short filaments containing one to three spores, were isolated and each thread drawn into a separate pipette and discharged into a separate test tube containing broth. All grew except three containing two, three, and two spores respec-

<sup>1</sup> *Jour. Infect. Dis.*, 1908, 5, p. 380; *Kansas University Sci. Bull.*, 1907, 4, p. 3.

tively. In another series, carried out by Dr. Hecker of this laboratory, 20 threads actively growing were manipulated in this manner and the emptied pipettes refilled with broth and incubated. The broth tubes or hanging drops receiving the discharge all showed growth while all the pipettes except two remained sterile. In a third series 15 single yeast cells, or attached groups of two to five, were treated in the same way as the anthrax filaments. Yeast cells were chosen because almost invariably viable when taken from actively growing cultures. Of these 15 all grew in the test tubes. So out of 50 experiments 47 exhibited growth in the test cultures. All experiments with actively growing threads or yeasts were positive, so it is not improbable that the failure of the three spore experiments was due to the lack of viability of the spores.

Further evidence of the reliability of the method is shown in portions of Table 1 where the material inoculated was especially virulent or the animals especially susceptible. In group 125, for instance, where all animals except one were inoculated with a single thread, four out of five were infected; and in the third inoculation of group 90 where larger doses were given, four out of four succumbed. The possibility of all or part of the inoculation material remaining behind cannot in every case be excluded, but the error due to this cause must be very small.

The chief sources of error in these experiments are, first, those inseparable from any animal experiments of this sort, the impossibility of exactly measuring the virulence of the inoculation material or of the varying resistance of the animals. These errors were eliminated to some extent by using a large number of animals. Over 160 mice were employed, not counting 23 which were inoculated with large initial doses for comparison or for furnishing inoculation material; and about 575 inoculations were made in the whole series. In the larger proportion of the inoculations material was taken directly from the blood or organs of an infected animal, in a majority of the cases after two or more passages through a series of animals. Material was taken from the animal and inoculated as soon as possible after death, and was kept cold until used, usually in a refrigerator. In some series material was inoculated within one or two hours after the death of the animal. In the culture and spore experiments nearly



uniform conditions of growth were observed. Animals were kept, so far as possible, under conditions favorable to health.

A special source of error as regards the inoculation material is incident to the use of very minute doses. Here the chances of variation are greater than when larger doses are employed and there is the further danger of selecting dead or degenerate organisms for inoculation. In order to minimize this error selection was made of plump, healthy-looking bacteria, the organisms were not allowed to remain long isolated in droplets before inoculation, and droplets were protected from drying and strong light. Generally only a small number of animals were inoculated, rarely more than five, before fresh droplets were made, usually taken from the cold emulsion of organs or blood in salt solution. Inoculations were made as rapidly as possible. In some instances where small doses were given, seven or eight inoculations were made in an hour.

A test of the viability of the material inoculated was furnished by leaving in the droplets the bacilli remaining unused after a series of inoculations. The subsequent growth of a large percentage of these bacilli on the cover glass showed that they were viable. It is possible also that bacilli taken directly from an organ and inoculated into a mouse would be more likely to grow than when left in the culture medium used, a mixture of salt solution and mouse serum.

The possibility of inoculating aggressin, or some material capable of promoting infection, was kept in view, and in some earlier series the bacilli were washed by centrifuging before inoculation. But the dilution in salt solution, and the very small amount of this dilution inoculated reduce to a minimum the possibility of variation in results from this source. In the series where washed bacilli were used, the course of events was much the same as in cases where inoculated material was simply diluted with salt solution. It was demonstrated that a bacillus  $6\mu$  long taken from the blood and washed in salt solution is capable of fatal infection.

All mice were inoculated at the same or approximately the same point—at one side of the root of the tail—and the dose was injected into the subcutaneous tissue, often somewhat beneath the skin itself. Since the point of the pipette could not always be brought into exactly the same place, some variation from this source as regards

infection cannot be excluded. The lesion made by the very fine point of the pipette is so small as to be scarcely visible under the lens.

In Table 1 are given the results of experiments made with virulent vegetative material, taken, with a few exceptions, directly from the cadavers of infected animals. The exceptions are chiefly those in which the first one or two doses were made with some other material, either spores or culture material in the vegetative form. A few are included in which the material for one or two doses came from an infected guinea-pig or from infected insects. All these exceptions are indicated in the table.

Mice are ranged in groups separated in the table by horizontal lines. In each group are arranged those animals which received the same, or approximately the same, treatment during at least a part of the period of inoculations. This is especially true of the groups below 132. In these groups inoculations carried out on the same dates and with material from the same cadaver are for the most part found in one vertical column. The members of the first and second groups have less in common and are arranged together for convenience. In practically every case a given inoculation material was used only on one day.

For convenience in reference a group will be referred to by its first number: thus group 2 includes numbers 2, 3, and 4.

The unit in dosage is one linear micromillimeter. The number of threads inoculated, indicated by the Roman characters, is followed in the table by a number giving the sum of the lengths of these rods or threads as measured by the eyepiece micrometer. The intervals between doses are somewhat irregular in the earlier inoculations owing to interruptions in the work, but in the later ones they were for the most part between 10 and 20 days. A sufficient time was allowed for possible hypersensitiveness to develop, and to make the interval far exceed the usual interval intervening between the last inoculation and death from anthrax. In a number of the later inoculations the interval was shortened to seven to eleven days.

In a number of cases inoculated animals died from causes other than anthrax, though for the most part animals remained in good health over long periods of confinement in individual cages. The cages had for a base a glass jar partly filled with sawdust and cotton

and, extending some six inches above this, an extension made of wire screen; so that the animals had roomy, well-ventilated quarters. It was observed that some animals did not do well immediately on removal to separate cages, especially in colder weather. To obviate a possible lowering of resistance from this cause animals in groups 125, 131, 139, 150, 171, and 181, aggregating nearly one-half of the total number in the table, were isolated some weeks before the first inoculation. The experiments extended over nearly one year, and no particular seasonal variation was observed.

It will be observed that the dose is made to increase slowly in groups 2, 83, 90, and some members of 13. It is made to increase rapidly in groups 171 and 181. It is made to remain nearly constant and relatively large in the large part of groups 19, 54, 71, 78, and 95. In some members of these groups an initial increase is followed by a slight decrease or the dose remains nearly constant. It is made to remain nearly constant and relatively small in groups 113, 125, 131, 139, 150, and 161. It decreases constantly in Nos. 65 and 95. In several groups the dose was made to increase rapidly at the end, and in groups 61, 64, 71, 78, 95, 181, and in Nos. 57, 58, and 59 a large initial dose of mouse or cockroach material was given. In group 181 an initial dose of mixt fresh and culture material was inoculated.

In none of these combinations do we find evidence of the establishment of any degree of immunity. Some mice resisted much longer than others and a few of these resistant ones were still surviving at the close of the experiments; but the resistance of these animals can well be explained by their greater natural immunity or by the size or condition of the animal without assuming the acquirement of artificial immunity. It will be observed that group 150, which consisted principally of large male mice, showed better resistance to the initial doses than the smaller animals in group 131, though the latter received smaller doses. Members of both lots had been isolated in individual cages for some weeks previous to the first inoculation. Also the mice of the more resistant groups 171 and 181 were older and in better condition than the mice of the same lot included in groups 113, 125, and 131. Further, it will be observed that the more resistant mice more often showed a decided

TABLE 1.

## MICE INOCULATED WITH MATERIAL DIRECTLY FROM INFECTED ANIMALS.

Under species, *w*=white, *g*=gray. In weight column first number or designation=weight at beginning of a series; second=weight at autopsy or at end of series. If two numbers in the first column, lower=weight at some time during series. Roman numerals=number of filaments, Arabic=total number of micromillimeters in dose. Thus II=27 means two filaments aggregating 27  $\mu$ . In second line of dose description letters b, s, l, k, and st indicate blood, spleen, liver, kidney, and subcutaneous fluid respectively. If culture, 1 br, 1 ag, 2 ag, etc., indicate first broth, first agar, second agar, etc., cultures respectively. sp=spore. Numbers indicate number of mouse from which inoculating material was taken. Thus b 34=blood of No. 34, etc. clcr=cockroach, gp=guinea-pig. Following last dose +A=died of Anthrax, +?=died cause unknown. Numbers in parentheses=number of mouse passages preceding material inoculated at fatal dose.

No.	Species	Weight in Grams	Dose in Micro-millimeters	Intervals—Days	Dose in Micro-millimeters	Intervals—Days	Dose in Micro-millimeters	Intervals—Days	Dose in Micro-millimeters	Intervals—Days	Dose in Micro-millimeters	Intervals—Days
2	w	med.	I=6 b 1	2	+A(1)	10	I=40 b 15	39	V=46 b 53	19	IV=50 b 16	15
3	w	med.-20.0	I=9 b 1	6	I=21 b 10	10						
3	w	(Continued)	+A(1)									
4	w	med.-20.0	I=30 b 1	7	I=18 b 9	9	I=40 b 15	39	VIII=52 b 53	16	III=65 b 63	18
4	w	(Continued)	+A(1)									
13	w	med.	I=9 b 10	10	II=27 b 15	4	+A(4)					
14	w	med.-22.0	I=7 b 10	10	II=27 b 15	39	VI=31 b 53	16	II=48 b 63	18	VII=54 b 82	4
16	w		I=6 b 9	48	II=13 b 53	16	II=42 b 63	2	+A(1)			
17	w	med.	I=21+1 sp 1 br 9	14	III=84+2 sp 1 br 13	42	10 sp b 14	2	+			
18	w	med.	I=36+1 sp 1 br 9	14	IV=84+2 sp 1 br 13	33	VII=84 b 53	3	+A(3)			
34	g	small	I=12 b 15	3	+A(4)							
37	g	med.	II=57 b 15	42	I=21 b 18	16	II=27 b 16	15	+			
40	g	med.-15.5	II=57 b 34	36	III=57 b 53	19	VI=57 b 16	15	VI=73 b 82	3	+A(1)	

19	W	10.0 (Continued)	+A(1)	I=12 b 7	47	I=15 b 53	35	II=25 b 78	17	V=26 b 98	23	III=36 b 105	2
19	W	med.-22.0 (Continued)	+A(1)	I=12 b gp	43	III=15 b 53	34	III=36 b 82	18	III=35 b 98	23	IV=48 b 105	4
27	W	med.-24.7 (Continued)	Large am't de- gen. rods	I=27 b 18	16	IV=35 b 16	15	VI=43 b 82	18	V=30 b 98	23	III=45 b 105	14
41	W	(Continued)	V1=45 b 110 +A(1)	V=40 b 24	14	V=40 b 125	18	III=41 b 26	16	III=43 s 156	24	IV=49 s 28	5
41	W	(Continued)	III=93+3 sp I br 13 X=63 b 110	I=18 b 18 +A(3)	16	II=30 b 16	15	IV=43 b 82	18	VI=44 b 98	23	IV=57 b 105	14
45	W	20.0 (Continued)		75-100 sp died 10	33	V=51 b 18	31	IV=69 b 82	18	X=63 b 98	23	V1=75 b 105	5
50	W	20.0 (Continued)	+A(1)	VIII=47 b 27	14	V1=43 b 92	5	+A(4)					
54	G	med.-15.9	I=9 b 11	VIII=25 b 78 +A(5)	19	II=26 b 99	21	II=30 b 105	14	V1=42 b 110	28	V1=38 b 24	14
55	G	med.-18.3 (Continued)	I=18 b 11 III=43 b 125	30	10	II=10 b 99	21	I=30 b 105	6	+A(1)			
55	G	med.-17	I=9 b 11	30	10	II=10 b 99	21	III=45 b 105	14	V1=55 b 110	28	V1=50 b 24	14
57	W	med.-20.3 (Continued)	XXIII=846 chr 10 +A(2)	20	10	III=34 b 99	21	IV=30 b 105	14	VII=54 b 110	28	VIII=60 s 158	14
57	W	med.-24.4 (Continued)	VII=117 chr 10 V1=50 b 125 IX=73 s 106	30	10	IV=30 b 99	21	IV=30 b 105	14	VII=54 b 110	28	V1=50 b 24	14
50	W	med.-27.5 (Continued)	CXCI=7028 chr 10 V1=63 s 156 VIII=100 s 36	74	14	V1=54 b 92	24	VII=50 b 24	14	V1=55 b 125	18	VII=65 b 26	16
58	W	large 25.8-27.5 (Continued)		24	14	V1=60 s 25	12	IX=67 s 158	11	IX=83 s 25	10	XI=88 s 185	11
58	W	(Continued)		8	7	XXI=337 s 178							





85	W	20.0	I=8 b 14	37	I=15 b 105	14	II=25 b 110	3	+A(3)				
86	W	25.5	I=8 b 14	37	II=18 b 105	14	III=24 b 110	3	+A(3)				
87	W		I=8 b 14	1	+								
88	W		I=8 b 14	3	+								
89	W	17.0	I=6 b 14	6	+								
90	W	26.0	II=28 13	33	III=41 119	13	V=56 1111	3	+A(3)				
91	W	23.2	II=18 13	33	III=32 119	13	V=45 1111	5	+A(3)				
92	W	25.0	II=12 13	33	II=20 119	13	VII=38 1111	3	+A(3)				
93	W	28.0	III=34 13	33	IV=46 119	13	VIII=65 1111	4	+A(3)				
94	W	21.0	III=27 13	32	+								
95	W	24.2	XXV=340 ckr 147	20	VI=52 b 27	14	VII=48 b 92	3	+A(4)				
96	W	22.0	XX=216 ckr 147	3	+A(1)								
97	W	25.5-26.7	XX=268 ckr 147	20	V=49 b 27	14	III=34 b 92	26	IV=34 b 24	14	V=39 b 125	5	+A(5)
98	W	23.0	XX=246 ckr 147	2	+A(1)								
99	W	21.0	XXVIII=390 ckr 1-7	4	+A(1)								
113	W	18 -13.0	I=9 b 95	5	+								
114	W	18.5-14.6	I=12 b 95	3	+								
115	W	15.5-12.8	I=9 b 95	3	+A(5)								
116	W	20 -15.1	II=14 b 95	2	+								



133	W	18.8-26.6 (Continued)	I=6 b 125 III=34 s 106	18	I=6 b 26 VI=46 s 185	16	I=6 s 56 VI=53 s 38	24	I=6 s 28 XVI=103 s 109	14	I=4 s 25 +A(7)	12	II=7 s 158	11
133	W			10		11		8		3				
135	W	12.7-18.0 (Continued)	I=4.5 b 125 V=37 s 106	18	I=5 b 26 +A(7)	16	I=5 s 156	24	I=6 s 28	14	I=7 s 25	12	I=7 s 158	11
135	W			3										
136	W	16.5-21.0	I=6 b 125	4	+A(5)									
137	W	15.3-17.1	I=4 b 125	6	+A(5)									
138	W	15.9-17.8	II=6 b 125	3	+A(5)									
139	W	18.2-10.2 16.3 (Continued)	I=3 b 74 IV=40 s 62	20	I=4.5 b 79 VI=64 s 185	14	I=5 s 84 IX=127 s 38	19	I=6 s 28 XXI=236 s 109	15	I=6 s 25 +A(7)	12	I=6 s 158	11
140	W	20.5-24.9 20.6 (Continued)	I=4 b 74 II=31 s 62	20	I=4 b 79 +A(1)	14	I=4 s 84	19	I=4 s 28	15	I=4 s 25	12	I=4 s 158	11
140	W			2										
141	W	20.-25.5 22.0 (Continued)	I=4 b 74 IV=51 s 62	20	I=4 b 79 +A(1)	14	I=4 s 84	19	I=3 s 28	15	I=3 s 25	12	I=3 s 158	11
141	W			3										
142	W	19.6-20.4 17.4	I=5 b 74	20	I=5 b 79	14	I=5 s 84	19	I=6 s 28	4	+A(1)			
143	W	19.5-20.6 18.7 (Continued)	I=5 b 74 IV=40 s 62	20	I=6 b 79 +A(1)	14	I=7 s 84	19	I=7 s 28	15	I=6 s 25	12	I=6 s 158	11
143	W			3										
144	W	19.-20.8 20.7	I=2 b 74	20	I=4 b 79	14	I=4 s 84	19	I=4 s 28	3	+A(1)			
145	W	17.4-16.9 16.2	I=3 b 74	20	I=3.5 b 79	3	+A(3)							
147	W	19.3 19.0	I=3+ b 74	20	I=9 b 79	14	I=7 s 84	19	I=6 s 28	15	+			

TABLE 1.—Continued.

[illegible]

[illegible]

TABLE 1.—Continued.

No.	Species	Weight in Grams	Dose in Micro-millimeters	Intervals between Doses—Days	Dose in Micro-millimeters	Intervals between Doses—Days	Dose in Micro-millimeters	Intervals between Doses—Days	Dose in Micro-millimeters	Intervals between Doses—Days	Dose in Micro-millimeters	Intervals between Doses—Days
183	w	18.5-22.0	III=42 S 31 IV=120 2 ag h 31 V=40 S 31 IV=144 2 ag h 31 II=45 S 31 VII=108 2 ag h 31 VI=53 S 31 VII=180 2 ag h 31 V=50 S 31 VIII=165 2 ag h 31	22	VIII=74 S 22	8	XIX=201 S 176	4	+A(4)			
184	w	10.2-10.4		22	VI=50 S 22	3	+A(2)					
185	w	15.6-16		22	XI=64 S 22	2	+A(2)					
186	w	18.8-17.8		22	VIII=64 S 22	8	XXI=179 S 176	8	XLVI=254 S 101	4	+A(6)	
187	w	23.5-10.2		22	VIII=66 S 22	3	+A(2)					



increase in weight during confinement. Some instances will be noted where mice succumbed to doses less than or equal to those which they had previously withstood.

So while the possibility of the acquirement of some artificial immunity cannot be excluded, all the variations noted can be explained equally as well or better by reference to the natural resistance of the animal.

As regards hypersensitization the evidence is also negative. In groups 113, 125, 131, 139, and 150 the same relatively small dose was repeated through many inoculations with especial reference to the determination of possible hypersensitizing. Here there was considerable mortality following the first inoculations, due presumably to the weeding out of the more susceptible individuals. Then followed a relatively slow rate of mortality with some individuals surviving many successive doses. Four out of nine members of group 150 survive seven inoculations, and in other groups also, where nearly constant doses of relatively larger size were given, some members survive many successive doses. The final infection and death of mice in these groups can be fully explained, it seems to me, by the possibility of doses of increased virulence, or by a falling off in the condition of the animal, without resorting to the assumption of hypersensitizing. For the relation between the number of the dose and the mortality see Table 5.

In Table 2 are grouped the results of experiments having the same aim as those of Table 1. Here spores are inoculated instead of fresh material. The spores, except in a few instances indicated in the table, were formed in the first culture from the animal, and were grown in a mixture of the culture medium and blood or organ extract from the infected animal. In the earlier experiments hanging drop cultures and a medium composed of a mixture of broth or salt solution with material from the infected animal were used. In later experiments blood or portions of organs were placed on peptone-free agar. Spores were in nearly all cases grown at 37° C. and inoculated soon after they were ripe, usually within 30 hours after planting. On one inoculation date spores dried for 17 days but still viable were inoculated. See \* in Nos. 5, 6, 47, 48, and 49 in the table.

Spores were inoculated free after the dissolution of the mother cell,



22	W	20.4	1 sp b 10	47	2 sp b 53	38	3 sp b 14	50	4 sp s 110	28	5 sp l 24	15	6 sp s 125	23	7 sp s 70	13	8 sp l 84	21
22	W	(Continued)	9 sp s 28	14	10 sp+12 l 25	13	11 sp s 158	10	12 sp l 62	7	+A(1)	7						
23	W		2 sp b 17	47	2 sp b 53	89	4 sp s 110	28	5 sp l 24	15	6 sp s 125	23	7 sp+3 s 70	13	8 sp l 84	21	9 sp s 58	14
23	W	(Continued)	10 sp+12 l 25	13	11 sp s 158	10	12 sp l 62	11	13 sp s 184	10	14 sp+9 l 38	8	15 sp l 109					
42	W	26.3	2 sp b 13	35	5-6 sp b 53	38	6 sp b 14	36	10 sp b 105	15	20 sp st 111	27	14 sp+27 l 24	4	+A(2)			
43	W	26.5			3 sp b 13	73	7 sp b 14	36	7 sp+6 b 105	15	13 sp st 111	27	15 sp l 24	15	20 sp+9 s 125	23	24 sp+20 s 70	4
43	W	(Continued)	+A(3)															
44	W	21.0	4 sp b 13	43	0 sp b 14	3	+A(2)											
47	W	28.0	15 sp* b 10	32	6 sp b 53	13	10 sp b 105	15	13 sp st 111	27	15 sp+6 l 24	15	15 sp s 125	23	20 sp+30 s 70	13	25 sp+50 l 84	21
47	W	(Continued)	30 sp+150 s 28	14	10 sp+120 l 25	13	45 sp s 158	10	56 sp	21	55 sp+100 l 38	8	117 sp l 109					
48	W	24.1	4 sp* b 10	32	0 sp b 53	74	10 sp b 105	15	12 sp+6 st 111	27	24 sp+30 l 24	15	20 sp+12 s 125	23	35 sp s 70	13	39 sp l 84	21
48	W	(Continued)	40 sp+120 s 28	14	10 sp+120 l 25	3	+A(1)											
49	W	20.0	1 sp* b 10	106	7 sp b 105	15	12 sp st 111	2	+A(4)									

\* Dried spores.

or, especially during later inoculations, taken when still in chains. It was found exceedingly difficult to take up loose spores when isolated in hanging drops of liquid media, since, once in contact with the layer of surface tension at the bottom of the droplet, they could scarcely be separated from it. The pipette draws off the liquid, while the spore follows the surface tension layer to the cover glass and remains there. In a large variety of bacilli and spores manipulated by this technic I have found the above mentioned characteristic shown in any marked degree by none except the anthrax spore and the tubercle bacillus. In some cases I have partly overcome the difficulty by using gelatin, soft agar, or some other semi-solid medium in place of salt solution or broth. Again, if the anthrax spore or tubercle bacillus be taken up soon after isolation, while still suspended above the layer of surface tension and still exhibiting the Brownian movement, there is little difficulty in making them enter the pipette. Here I have found the double pipette holder of service, the bacillus or spore being taken up by a second pipette soon after its isolation by the first.

In some cases, indicated in the tables, filaments not containing spores were inoculated also. The amounts were small, and, considering that the threads were from a relatively old culture and probably somewhat degenerate, it is not likely that they had any effect on infection. In practically all mice of the spore series the dose was made to increase gradually. In No. 22 and the greater part of the histories of Nos. 5 and 23 the most gradual increase possible is maintained, namely one spore at a time. The results of experiments given in Table 2 are substantially the same as those of Table 1 and give no conclusive evidence of immunization or hypersensitization. (Compare Table 5.)

Table 3 contains the results of experiments with fresh cultures. With the exception of Nos. 188-191 cultures were made on agar and the number of removes from the animal are indicated on the table. Under "2ag," which includes the greater number of cases, infected blood or portions of organs were placed on the lower portion of agar slants and allowed to grow over night at 37°. Then the water of condensation, containing actively growing threads, was washed over the still sterile upper part of the slant of the same tube and allowed

to grow, usually five or six hours, until a layer of new growth had formed. This fresh growth was used for inoculation. Altho grown in the first tube from the infected animal, this is practically equivalent to the second agar culture. This procedure was followed in order to have filaments for inoculation that were living and rapidly growing.

In group 106 a few are included in which some spores were mixt with the vegative filaments and indicate a somewhat older culture.

It is seen in Table 3 that animals withstand a much larger initial dose of anthrax from culture than from infected tissues. This dosage was increased to over 2,000 units in group 100 before any animals became infected. Then No. 103, which, it will be noted, had lost considerable weight, succumbed. In order to test the resistance of the survivors fresh virulent material was substituted for culture material at the 12th inoculation and the dosage increased until all had yielded. On comparing these lethal doses with those found under groups 171 and 181 of Table 1, it will be seen that the preliminary treatment with culture material had conferred very little, if any, immunity against fresh material, altho possibly these mice had attained some immunity against culture material.

In Nos. 188-191 virulent material was sown in hanging drops in a medium of salt solution mixt with spleen pulp. The growth of 24 hours at room temperature was inoculated. The results show that bacilli, tho taken directly from the animal and allowed to increase in an extract of an infected organ, soon lose much of their power of infection.

Up to doses of 500 to 600 units filaments were measured separately, as in doses given in Table 1. In larger doses the quantity was estimated by measuring, in droplets containing a few threads, the units in one or two filaments of suitable size. The total number of units in the droplet was then estimated by using the measured filaments as a standard. A few of the larger doses in Table 1 were estimated in this way.

As regards the bearing these experiments have on the subject of infection, reference may be made, first, to Table 4. Here the infectivity of anthrax taken directly from an infected insect instead of from a mouse may be estimated. The material was obtained by inoculat-

TABLE 3.  
MICE INOCULATED WITH MATERIAL FROM CULTURES.  
Dose = material grown in hanging drops in mixture of mouse serum and salt solution.

[illegible]





ing cockroaches in the abdomen or leg by means of a fine-pointed capillary pipette. When large doses were given the animals usually succumbed to anthrax in 24 to 48 hours, whether kept at incubator or

TABLE 4.  
MICE INOCULATED WITH MATERIAL FROM INFECTED COCKROACHES.

No.	Species	Weight in Grams	Dose in Micro-millimeters	Intervals between Doses—Days	
66	g	med.	XXXIX=624 ckr 54	2	+A(1)
67	g	med.	XXIV=453 ckr 54	2	+A(1) *
68	g	med.	LXVI=1044 ckr 54	3	+A(1)
69	g	small	XXXIX=720 ckr 54	2	+ ?
70	w	large	LXXXIX=1136 ckr 54	4	+A(1)
57	w	med.-20.3	XXIII=846 ckr 10	29	*
58	w	large-25.8	CXCI=7028 ckr 10	74	*
59	w	med.-24.4	VII=117 ckr 10	30	*
78	w	large-25.4	XX=261 ckr 107	3	+A
79	w	large-21.8	XXV=366 ckr 107	48	*
80	g	large-15.6	XXIII=236 ckr 107	3	+A
81	w	large	XXV=379 ckr 107	48	*
95	w	24.2	XXV=340 ckr 147	29	*
96	w	22.0	XX=216 ckr 147	3	+A
97	w	25.5-26.7	XX=268 ckr 147	29	*
98	w	23.0	XX=246 ckr 147	2	+A
99	w	21.0	XXVIII=390 ckr 147	4	+A

\* For subsequent doses see Table 1.

room temperature. It is observed that this material of insect origin is somewhat less infective for mice than that of mouse origin. (Compare Table 6.) The bacilli from cockroaches were, for the most part, capsulated as shown by staining and by observing them mounted

in India ink. These results would indicate that it is not the capsule alone which determines the greater infectivity of bacilli of animal origin else we would expect the cockroach material to be as infective as the mouse.

Below No. 70 in this table are given a group of 12 taken from Table 1, the initial doses of which are repeated for comparison.

In Table 5 under each dose number is shown the total number of mice which reached this dose number, and the number and percentage dying of anthrax. Thus of the 68 which received a second dose, 10, or 15 per cent, died. Of the virulent vegetative material only doses of 100  $\mu$  or less are included, and in the spore series only doses of 30 spores or less. It will be noted that there is no tendency to an increase or decrease of mortality until the eighth dose

TABLE 5.  
RELATION BETWEEN NUMBER OF DOSE AND NUMBER OF MICE DYING.  
Doses 100  $\mu$  or less and 30 spores or less.

Number of Dose Virulent Vegetative	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Number inoculated.....	83	68	55	35	30	23	19	10	5	4	3	3	2	0
Number dying.....	20	10	15	4	7	3	0	1	0	0	0	1	1	0
Percentage dying.....	24	15	27	11	23	13	47	10	0	0	0	33	50	0
Number of Dose Spore Series														
Number inoculated.....	18	13	12	0	8	8	5	5	4	3	3	3	1	1
Number dying.....	4	1	3	0	0	2	1	0	0	0	0	1	0	0
Percentage dying.....	22	7	25	0	0	25	20	0	0	0	0	33	0	0

is reached, when the few survivors show considerable resistance. This is the more noteworthy since the doses beyond the seventh inoculation were greater than 30 microns or nine spores. If the interpretation of results given above is accepted we have here the phenomenon sometimes observed in severe epidemics—the survival of the few exceptionally well endowed with natural resistance. If hypersensitiveness were being developed we would expect a progressive increase in the percentage mortality.

In Table 6 the mouse numbers and the percentages dying of anthrax are arranged under doses of different magnitude instead of under dose number. Doses of all magnitudes are considered and results are arranged according to the source of the inoculation material. The smallest lethal dose was observed in No. 145, where the fatal dose,  $3\frac{1}{2}$   $\mu$ , presumably one bacillus, was taken from the liver of an

infected mouse. In another case (No. 7) the lethal dose was two spores. The increased mortality with the larger doses is observable. The relative infectivity of material from the different sources is also shown. Roughly estimated, material directly from the infected

TABLE 6.  
SIZE OF FATAL DOSES AND NUMBERS DYING AT EACH QUANTITY.  
VEGETATIVE.

MICRO- MILLIMETERS	FRESH FROM INFECTED MICE			CULTURE			FRESH FROM INFECTED COCKROACH		
	Total No. Inocu- lated	No. Dying	Percent- age Dying	Total No. Inocu- lated	No. Dying	Percent- age Dying	Total No. Inocu- la.ed	No. Dying	Percent- age Dying
1-5.....	41	3	7½						
6-10.....	88	17	19						
11-15.....	36	6	17						
16-25.....	18	5	27						
26-35.....	29	4	13						
36-50.....	63	15	23						
51-75.....	63	19	30						
76-100.....	11	3	27						
101-150.....	9	3	33				1		
151-200.....	7	2	28	3					
201-300.....	13	5	38	2			5	4	80
301-400.....	6	4	67	6			4	1	25
401-600.....	1	0	0	7			1	1	100
601-800.....				15	2	13	2		
801-1000.....				9			1		
1001-2000.....				16	2	12	2	2	
2001-.....				4	1	25	1		

mouse is nearly 100 times as infectious as material from fresh cultures. The number receiving cockroach material is too small to form a basis for a numerical estimate, but this material is evidently somewhat less infectious for mice than material of mouse origin.

Table 7 is similar to Table 6 except that spore doses are considered. Doses of all sizes are included.

TABLE 7.  
SIZE OF FATAL DOSES AND NUMBERS DYING AT EACH QUANTITY.  
Spores.

Dose No. of Spores	2	3-5	6-8	9-11	12-14	15-18	19-25	26-30	31-45	46-50	50+
Number inoculated...	8	18	15	14	12	9		6	10	4	5
Number dying.....	1	3	1	1	4	2		1	1	2	1
Percentage dying.....	12	16	6	7	33	22		16	10	50	20

There seems to be no definite relation between mortality and the number of threads or segments composing a dose of given size. Summarizing the data furnished by the Roman numerals in Table 1 we find out of a total of 78 dying of anthrax, 25 received one thread

in the lethal dose; 9 received two; 5, three; 6, four; 8, five; 5, six; 12, seven to fifteen; and 9, sixteen and above. Of 26 receiving fatal doses of  $15 \mu$  or less, 22 received but one thread.

As regards the virulence of the inoculated material, so far as it can be measured by the number of consecutive mouse passages, the following data, summarized from Table 1, are included in Table 8.

TABLE 8.  
VIRULENCE, AS SUMMARIZED FROM TABLE 1.

Inoculation Material No. of Mouse Passages	Total No. Mice Inocu- lated	No Dying of of Anthrax	Percentage Dying of Anthrax
1.....	78	23	29
2.....	33	9	27
3.....	46	17	37
4.....	55	12	22
5.....	37	15	40
6.....	14	2	14
7.....	16	3	19

As regards the portion of the infected animal from which the inoculated material was taken, the following data are summarized from Table 1: Blood, total number of inoculations, 183, mortality from anthrax, 24 per cent; spleen, total inoculations 176, mortality 17 per cent; liver, total inoculations 23, mortality 26 per cent. The spleen furnished a larger proportion of the small doses than the other organs.

The intervals between the last dose and death of the mice in Tables 1, 2, 3, and 4 are given in Table 9. For comparison are also given

TABLE 9.

Intervals in Days	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Totals
Mice from tables.....	0	16	57	26	11	4	3	0	2	0	0	0	0	1	116
Mice receiving mas- sive doses .....	14	5	1	2	0	0	0	0	1	0	0	0	0	0	23

the intervals following very large doses given to 23 mice not included in any of the above tables. These large doses were about  $\frac{1}{10}$ – $\frac{1}{20}$  cm. emulsion of organs or of cultures.

A histological examination was made of about 85 mice. As a rule, spleen, liver, kidney, and, in a considerable number of cases, tissues taken from beneath the point of inoculation, were sectioned in paraffin and stained by the Weigert method. In by far the greater number of those which died of anthrax the usual distribution of bacilli

was found in the organs; but in some the spleen, usually showing the largest number, had very few (in the spleen of No. 141 none could be found), while other organs, and especially the point of inoculation, showed them more abundantly. There seemed to be no relation between the numbers and distribution of bacilli and the dosage or other treatment of the animals, tho animals of the spore series seemed to show somewhat greater numbers in the organs.

Large masses of bacilli were often found in the tissues at the point of inoculation, and near these were frequently found collections of phagocytes. In the masses of phagocytes bacilli were often granular and apparently degenerate. This condition was found in animals which had received but one dose as well as in those which had received several and could not therefore be regarded as a sign of acquired resistance.

Sections were made of nearly all those which died of some cause apparently other than anthrax, and cultures were made from the organs of some doubtful ones. It is probable that, owing to the possibility of missing some small focus of infection, some of these animals should be included among those which died of anthrax, especially when the interval following the last dose was two to six days.

Considerable edema was observed at or near the point of inoculation of many at autopsy. This was found in those which had received but one dose, as well as in those which had received several, and so could have no relation to acquired immunity.

Considering the difference in size, the gray mice reacted about the same as the whites as regards infection and immunity. A guinea-pig inoculated with five spores died of anthrax after a five days' interval.

In conclusion it may be stated that these experiments give no ground for the assumption that any degree of immunity or hypersensitiveness can be conferred on the mouse by repeated minute doses of anthrax, either in the vegetative stage taken directly from the infected animal, in the form of virulent spores, or in the vegetative form somewhat attenuated by cultivation. The conditions imposed on these experiments, the combination of high virulence with high susceptibility, are severe; and results might be otherwise in the



case of another animal or microorganism where either or both factors might be materially altered.

As regards the lethal dose of material from infected organs a bacillus  $3\frac{1}{2} \mu$  long was, in one case, found to be a fatal dose; and it is evident that a sublethal dose, under these conditions, does not exist for a very susceptible individual. Fresh virulent material from the mouse has for mice about 100 times the infectivity of material grown in the first agar or broth tube, and a somewhat greater infectivity than that of capsulated bacilli directly from infected cockroaches.

# ON THE EFFECT OF REACTION AND OF CERTAIN SALTS ON NORMAL OPSONINS.\*

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## I. ON THE EFFECT OF REACTION ON OPSONIFICATION BY NORMAL SERUM.

It would be natural to expect that normal opsonins would display their maximum activity at the normal reaction of the blood, that is, at a reaction slightly alkaline to the ordinary indicators. Noguchi,<sup>†</sup> however, obtained results to the effect that the opsonins of normal serum act best in a neutral medium, and that any acidity or alkalinity

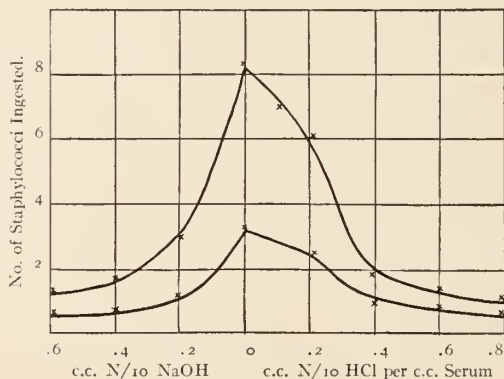


CHART 1.—Effect of reaction on opsonin of dog serum.

results in diminished opsonification. He pointed out a difference in behavior between opsonin and the complement of normal serum, the latter being most effective in an alkaline medium. For various reasons it seemed desirable to repeat some of the work of Noguchi, and this I have done, but without reaching the results that he did.

The technic used by me was as follows: 24–48 hour growths of *Staph. albus* on agar were suspended in salt solution, washed once, and resuspended in small quantities (about 1 c.c.) of salt solution. Such suspensions gave no acid reaction with lacmoid paper. Mixtures were then made by adding to equal quantities of fresh serum varying amounts of N/10 acid (HCl), or alkali (NaOH). The mixtures were then adjusted to a minimum constant volume with salt solution and incubated for half an hour at

\* Received for publication July 6, 1909.

<sup>†</sup> *Jour. Exper. Med.*, 1907, 9, p. 453.

37°. Then to each was added a given volume of the staphylococcus suspension and the whole incubated for half an hour. At the end of that time the cocci were thoroughly removed, using an extremely rapidly rotating "haematocrit" centrifuge apparatus, washed once, and finally resuspended in salt solution. These suspensions were then mixt with washed human leukocytes and opsonic counts made after the usual fashion. As it was feared that the final suspensions of organisms might not be of uniform density, in several instances bacterial counts of these suspensions were made. Such counts, when made, are given in the following tables.

Dog, rabbit, and human sera were used. The results appear in the following tables, the figures being the average count for at least 50 leukocytes:

## A. DOG SERUM.

		PHAGOCYTOSIS	
		Dog A	Dog B
1.	0.5 c.c. serum + 0.5 c.c. NaCl sol.	3.13	8.1
2.	" " " + 0.1 " N/20 HCl + 0.4 c.c. NaCl sol.	...	7.0
3.	" " " + 0.1 " N/10 " + 0.4 " " "	2.41	6.0
4.	" " " + 0.2 " " " + 0.3 " " "	1.20	1.84
5.	" " " + 0.3 " " " + 0.2 " " "	0.94	1.30
6.	" " " + 0.4 " " " + 0.1 " " "	0.62	1.00
7.	" " " + 0.5 " " " " " "	0.36	...
8.	" " " + 0.1 " " NaHO + 0.4 " " "	1.10	3.0
9.	" " " + 0.2 " " " + 0.3 " " "	0.70	1.6
10.	" " " + 0.3 " " " + 0.2 " " "	0.54	1.3

## B. RABBIT SERUM.

1

		Phagocytosis	Staph. per cu mm.
1.	0.1 c.c. serum + 0.24 c.c. NaCl sol.		
2.	" " " + 0.08 " N/10 HCl + 0.16 c.c. NaCl sol.	11.4	510,000
3.	" " " + 0.16 " " " + 0.08 " " "	4.2	540,000
4.	" " " 0.24 " " " " " "	3.1	600,000

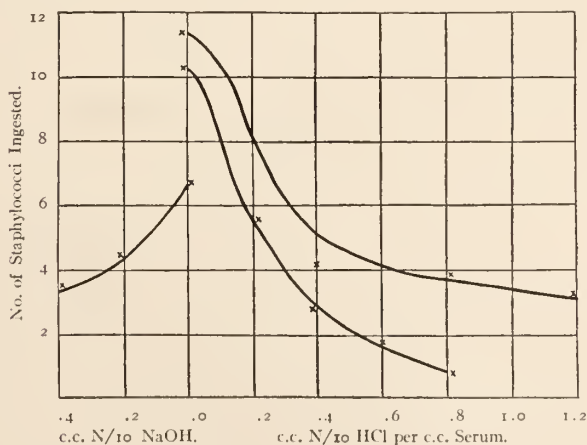


CHART 2.—Effect of reaction on opsonin of rabbit serum.

2

1.	0.1 c.c. serum + 0.08 c.c. NaCl sol.	10.2	526,000
2.	" " " + 0.02 " N/10 HCl + 0.06 c.c. NaCl sol.	5.6	637,000
3.	" " " + 0.04 " " " + 0.04 " " "	2.9	657,000
4.	" " " + 0.06 " " " + 0.02 " " "	1.6	634,000
5.	" " " + 0.08 " " " " " "	0.8	533,000

1.	0.1 c.c. serum + 0.08 c.c. NaCl sol.	3	6.6	.....
2.	" " " + 0.02 " N/10 NaHO + 0.06 c.c. NaCl sol.		4.3	.....
3.	" " " + 0.04 " " " + 0.04 " " "		3.42	.....

## C. HUMAN SERUM

		PHAGOCYTOSIS			
		"E"	"M"	"J"	
1.	0.1 c.c. serum + 0.08 NaCl sol.	I	2		
2.	" " " + 0.02 " N/10 HCl + 0.06 c.c. NaCl sol.	4.70	3.01	27.1	18.5
3.	" " " + 0.04 " " " + 0.04 " " "	3.12	3.56	25.2	.....
4.	" " " + 0.06 " " " + 0.02 " " "	3.30	2.59	22.7	16.9
5.	" " " + 0.08 " " " " " " "	1.20	0.70	4.34	5.72
6.	" " " + 0.09 " " " " " " "	0.90	.....	.....	1.76
			0.52	.....	.....

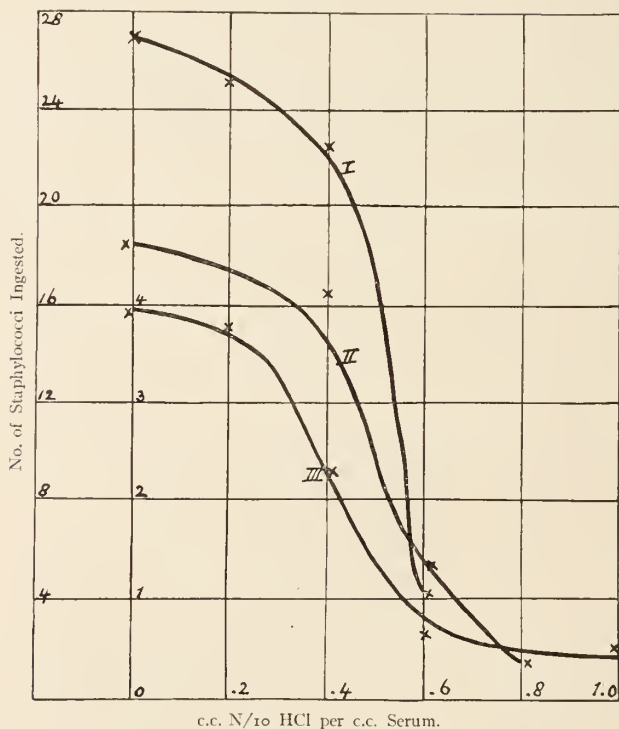


CHART 3.—Effect of reaction on opsonin of human serum: I, serum "M;" II, serum "J;" III, serum "E."

With the procedure used the results might have been influenced on the one hand by the effect of the acid (or alkali) on the organisms themselves, and on the other by the possibility of sufficient residual acidity remaining in the final suspension to unfavorably affect phagocytosis by action on the leukocytes. Hamburger and Hekma<sup>1</sup> have shown that a very slightly acid reaction is sufficient to inhibit phagocy-

<sup>1</sup> *Biochem. Ztschr.*, 1908, 9, p. 275.

tosis, in this manner, to a marked degree. To ascertain to what extent these objections would apply, the following control experiments were made:

#### I. EFFECT OF ACIDITY ON ORGANISMS.

1.	0.18 c.c.	NaCl sol.	+0.1	c.c.	staph. suspension	
2.	0.10	"	"	+0.08	" N/10 HCl	+0.1 c.c. staph. suspension
3.	0.14	"	"	+0.04	" NaHO	+0.1 " " "

These mixtures were incubated 30 minutes, washed once, and the staphylococci resuspended in equal volumes of salt solution; opsonic counts were made, using for the suspension equal volumes of these bacterial suspensions, rabbit serum, and (human) leukocytes.

1. gave an average count of 11.
2. " " " " " 7.7.
3. " " " " " 8.3.

It will be seen that while some diminution in phagocytosis resulted, the effect is not nearly as striking as is the effect of altered reaction on the serum itself.

#### 2. EFFECT OF RESIDUAL ACIDITY ON LEUKOCYTES.

Staphylococci were sensitized by incubation in serum+salt solution, just as in the first mixture of each of the tables, and were washed free from serum as in those experiments. To 0.1 c.c. of serum was added 0.08 c.c. N/10 HCl and 0.1 c.c. of salt solution. This was placed in one of the small centrifuge tubes used in the preceding work, and enough was drawn off to have a remainder equal to that ordinarily left in the tube in this work. An amount of salt solution equal to that used in washing the organisms in the previous work was now added, and after mixing the solution was again drawn off to the same point. The remainder was then taken up in the same volume of salt solution as was used in the final suspensions of the organisms. A mixture was made up of one part of this, one part of the sensitized, washed cocci, and one part of leukocytes suspension; as a control, a mixture of salt solution, sensitized organisms, and leukocytes was used. Opsonic counts gave for the former 2.6, for the control 2.04, thus indicating that what acid may have remained in the final mixture was too slight to affect the leukocytes.

One series of determinations was made to ascertain the effect of reaction on the streptococcus opsonin of dog's serum. The procedure was exactly that used for the work on staphylococcus.

1.	0.1 c.c. serum	+0.08 c.c. NaCl sol.	1.79
2.	" "	+0.02 " HCl +0.06 c.c. NaCl sol	1.66
3.	" "	+0.04 " " +0.04 " " "	1.10
4.	" "	+0.06 " " +0.02 " " "	0.68
5.	" "	+0.08 " " "	0.48

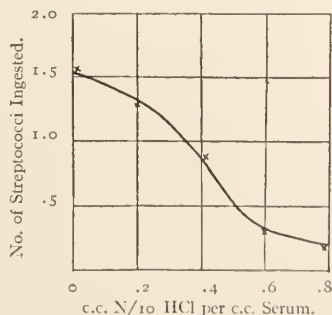


CHART 4.—Effect of reaction on streptococcus opsonin of dog serum.

To determine whether or not the effect of altered reaction on the thermolabile constituent of normal





measure at least due to its action on the thermolabile constituent of the opsonin.

## II. THE EFFECT OF CERTAIN SALTS ON PHAGOCYTOSIS.

Hektoen and Ruediger<sup>1</sup> found that a considerable number of salts, in sufficient concentration, would inhibit phagocytosis of bacteria to a greater or less degree. In no case did they find, with the concentrations used, any evidence of stimulation to greater phagocytic activity. This inhibitory effect of salts on phagocytosis they attributed at that time to action on the opsonins rather than on the leukocytes directly.

Hamburger and Hekma,<sup>2</sup> in an extensive study of the influence of various factors on phagocytosis, found that various salts, notably sodium fluorid and barium chlorid, had a marked inhibitory effect on phagocytosis, this action being exerted directly on the leukocytes. With calcium chlorid, on the other hand, they found the phagocytic power of the leukocytes for finely divided carbon particles markedly increased.

My own work has been done mainly with salts normally occurring in serum, a few additional ones being studied. At first staphylococci were treated with serum to which had been added the salt studied and then washed free from the modified serum before subjecting them to phagocytosis. These experiments, however, failed to establish any effect whatever of the salts on phagocytosis, with the exception of barium chlorid and sodium fluorid, and the inhibition obtained with sufficiently large amounts of these salts could quite probably be explained on the basis of absorpition of some of the salt by the cocci.

The plan was then adopted of adding the salt directly to the mixture of organisms, serum, and leukocytes. In this way, any action of the salt on any of the factors entering into the combination might be shown and the exact point of action determined later. The salts used were potassium sulfate, calcium, magnesium, barium, chlorids, and sodium fluorid in approximately  $m/8$  solution. Barium chlorid and sodium fluorid were found to exert a marked toxic effect on the leukocytes, in even very dilute solutions, so that the numerical presentation of the results obtained with them would not be of value.

The results obtained with the other salts are given below in tabular

<sup>1</sup> *Jour. Infect. Dis.*, 1905, 2, p. 128.

<sup>2</sup> *Biochem. Ztschr.*, 1908, 9, p. 275.

form. The exact strengths of the solutions were ascertained by titration with eighth normal silver nitrate solution.

Mixtures were made of equal parts of normal human serum, suspension of human leukocytes, suspension in physiological salt solution of a 24-hour growth of staphylococci, and solution of the given salt. For the lesser quantities of the salt examined the original solution was diluted with physiological salt solution. The fractions at the head of the columns represent the degree of dilution in any given case. The results are given in ratio of organisms ingested in the mixture containing the salts studied to that of those ingested in mixtures in which the serum was diluted to the same degree with physiological salt solution.

	DILUTIONS.				
	0	$\frac{1}{8}$	$\frac{1}{4}$	$\frac{1}{2}$	$\frac{1}{1}$
m/8 $K_2SO_4$ .....	1.0	0.81	1.02	1.01	0.93
m/8 $CaCl_2$ .....	1.0	1.0	1.08	1.06	0.50
1.3m/8 $MgCl_2$ .....	1.0	1.4	1.38	1.22	0.52

From these results it would appear that the only one of the three salts to have any marked effect in moderate concentrations on the phagocytosis of staphylococcus is magnesium chlorid, and that the action of this is stimulatory.

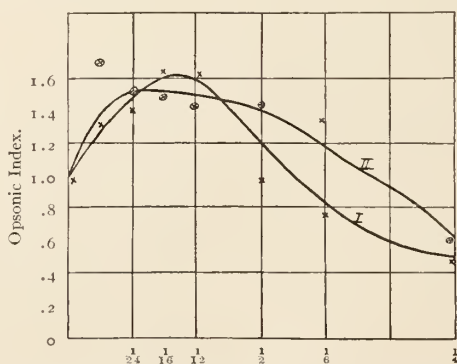


CHART 5.—Effect of  $MgCl$  on staphylococcus opsonin in human serum: I, staphylococci previously sensitized; II, staphylococci not previously sensitized.

Using magnesium chlorid again and a m/8 solution of strontium chlorid the following results were obtained:

	DILUTIONS.						
	0	$\frac{1}{8}$	$\frac{1}{4}$	$\frac{1}{2}$	$\frac{3}{4}$	$\frac{1}{1}$	
1.3m/8 $MgCl_2$ .....	1.0	1.70	1.53	1.50	1.44	1.46	0.64
m/8 $SrCl_4$ .....	1.0	0.78	0.96	0.97	0.90	1.22	1.10

The results here obtained with magnesium chlorid again show a stimulatory action of this salt on phagocytosis, while strontium chlorid, like the salts of the preceding series, is apparently practically inert.

To ascertain whether the action of the magnesium chlorid was on the leukocytes themselves or on the serum, the following experiment was made:

Staphylococci were sensitized by treating them with normal human serum for 30 minutes. They were then washed and resuspended in physiological salt solution. Mixtures were then made of equal parts of this suspension, of human leukocytes, of solution of the salt under examination, and of physiological salt solution. In this way the salt examined was present in dilutions corresponding exactly to those used in the preceding experiments. Magnesium and strontium chlorids were studied in this way.

		DILUTIONS.						
	0	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{128}$
1.3m/8 MgCl <sub>2</sub> .....	1.0	1.32	1.40	1.64	1.58	0.89	0.76	0.51
m/8 SrCl <sub>2</sub> .....	1.0	1.08	1.16	1.22	1.05	1.08	1.08	1.01

Here again, the magnesium chlorid acting only on the leukocyte exerts a stimulatory effect. So that, altho the graphic representation of the two sets of results does not show an absolute parallelism between the curves, it is very probable that the action of the salt is mainly at least on the leukocyte. Strontium chlorid, as before, is apparently without much effect.

It will be noted that the concentration of the magnesium chlorid solution is such as to heighten the osmotic pressure of the medium in which the leukocytes were finally suspended. Inasmuch as Hamburger and Hekma<sup>1</sup> found that comparatively slight increases in osmotic tension resulted in diminished phagocytic activity on the part of the leukocytes, the explanation of the apparent stimulation by the magnesium chlorid cannot be sought on the basis of altered tension.

From these results it would appear that as regards the phagocytosis of staphylococci by normal leukocytes in normal serum, the salts tested are for the greater part inert. Exceptions to this are barium chlorid and sodium fluorid, which exert a very toxic influence, and magnesium chlorid, which would appear to have some stimulatory action on the leukocyte.

<sup>1</sup> *Zitlingsverlag der Koninkl. Akad. v. Wetensch.*, 1907.

## SUBACUTE PLAGUE IN MAN DUE TO GROUND SQUIRREL INFECTION.\*

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AND

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THE subject of this report was a boy (J. M.) born in California of Portuguese parents. His age was 13 years and he was rather more than ordinarily well developed physically for his age. There was nothing in the previous history, medical or otherwise, having any bearing on the present illness, with the exception of the fact that, according to his own statement, he had been shooting ground squirrels in Alameda County, California, about a week before he was taken sick. According to the father's statement the boy had been out squirrel hunting just five days prior to his becoming ill. While on this hunting trip he shot four or five squirrels which he brought home with him. He stated that he had seen sick squirrels in the same vicinity, but did not shoot nor handle any of them.<sup>1</sup> This boy had not been more than a few miles from his home in his life and indeed had never seen a trolley car until he was brought to the hospital in Oakland. There is no evidence that he was in any place where he might have been infected with plague except on this hunting trip. There had been no known previous case of plague in man in California for nearly a year, and none in rodents, except ground squirrels, for about seven months.

The boy became sick on Tuesday, July 27, with headache and violent vomiting. The vomiting was almost continuous for about 24 hours. The next day he was seen by a physician and was found to have a temperature of 104° F. On Thursday, 48 hours after the beginning of the illness, the glands in the left axilla were found to be enlarged and tender. The patient was taken to a hospital in Oakland,

\* Received for publication, October 26, 1909.

<sup>1</sup> Since the above was written a plague infected squirrel has been shot in the immediate vicinity of where the boy had been hunting.—G. W. M., W. B. W.

California, where the glands in the left axilla were excised and found to be "broken down." They were not submitted to microscopical or bacteriological examination. It was distinctly stated that no pus was present.

The temperature did not fall materially after the glands were removed, but on the following day was about 104° F. On the second day after the operation it ranged between 99° and 102°. On Sunday, the third day after the operation (the fifth day of the illness), it was 102° F. On this day all of the superficial glands were markedly enlarged and tender, those of the neck and groins being especially prominent. Suspicion that the boy might be suffering from plague was now aroused. Information of the case was communicated to Surgeon Rupert Blue, commanding anti-plague operations in California, and by his instructions we investigated the case. At this time the boy's expression was anxious, his face flushed, his breathing rather rapid, pulse about 120, temperature 102° F., and he had the general appearance of being very sick. The cervical and inguinal glands were enlarged, fused together, and tender. The clinical picture was one characteristic of plague, and with the previous history did not leave one in any serious doubt as to the diagnosis. The left inguinal gland was excised with aseptic precautions and the tissues submitted to us and to Drs. Nusbaumer and Archibald for microscopical and bacteriological examination. Smears from the inguinal gland which had been excised on this day for diagnostic purposes showed numerous bacilli, which in shape, size, and staining reaction were identical with *B. pestis*.

Cultures made from these glands on agar gave a translucent sticky growth quite characteristic of *B. pestis*, and sub-cultures on salt agar gave well marked involution forms after 18 hours. Stalactites were developed in the broth culture and the reactions on the other media were those of *B. pestis*. Two guinea-pigs were inoculated, one by the cutaneous method; this animal died on the seventh day with characteristic lesions of plague and from its tissues *B. pestis* was recovered in pure culture. The other guinea-pig was inoculated subcutaneously, and died on the fifth day. Both had characteristic lesions of plague and from each one pure cultures of *B. pestis* were recovered. A white rat was inoculated by the subcutaneous

method with a piece of the gland and died on the third day with characteristic lesions of plague and a pure culture of *B. pestis* was isolated from its liver. Two white rats were vaccinated, that is, inoculated by the cutaneous method; one died on the fourth day, presenting the usual lesion of plague in rats. *B. pestis* was isolated from its liver. The other rat was killed on the 12th day and was found to be normal.

The boy was isolated at the Alameda County Infirmary during the next ten days; the temperature ranged from 100° F. to 103° F. He was given 40 c.c. of Yersin's anti-plague serum daily, but no very material change took place in his condition until the 10th day of the illness when a pustular eruption appeared which was uniformly distributed over the body. The pustules varied in diameter from 2 mm. to 10 mm. and each one was at first surrounded by a red areola. Pus taken from one of these lesions on the 12th day of the disease showed no bacteria in smear preparations but a guinea-pig inoculated subcutaneously with the pus died on the third day with characteristic lesions of early plague, and a pure culture of the plague bacillus was isolated from its liver. Another guinea-pig vaccinated with the same pus was killed on the seventh day and found to be entirely free from any lesions. As early as the fifth day of the illness the sputum was slightly tinged with blood, but it bore no resemblance to the sputum in pneumonic plague. This tinging with blood persisted only a day or two. On the 12th day of the illness a rather irregular area of dulness was found at the base of the left lung by the attending physician. On the 13th day marked exophthalmos appeared but without increase of ocular tension. Small tubercle-like bodies appeared on the iris at the margin of the left pupil. The exophthalmos became extreme, the breathing rapid, the pulse fast, and the boy died on the 16th day of the illness.

#### POST MORTEM FINDINGS.

The body is somewhat emaciated; the skin of a brownish and slightly yellowish color; post-mortem lividity of the dependent portions of the trunk and neck is fairly well marked; sclerae intensely icteric; the anterior edges of the cornea of both eyes show a few irregular petechiae and in the ocular conjunctiva above the left eye



are two submucous hemorrhages about 3 mm. in diameter; the anterior chamber of the right eye is somewhat cloudy; the pupils are irregular and in the iris near the edge of the left pupil are a few yellowish nodules 0.5-1 mm. in diameter. There is a bloody discharge from the right ear and a purulent one from the left.

There is a marked swelling on both sides of the neck, extending from below the ears to the region of the cervical glands; on section these glands are seen to be almost completely broken down into a pinkish purulent material.

There is an operation wound in the left axilla and another in the left femoral region; the right axillary glands are just palpable; there is a prominent bubo in the right femoral region and on section the right and left femoral and inguinal glands are seen to have undergone a necrosis similar to that of the cervical glands. The epitrochlear glands are palpable.

The muscles are dark red on section. Both pleural cavities are about half full of a clear amber colored fluid. The lungs are voluminous. Scattered irregularly throughout the substance of all the lobes of both lungs are many irregular tumor-like nodules which are of a light yellowish color. These vary in size from 2 mm. to 5 cm. in diameter. They are raised slightly above the pleural surface, feel hard on pressure, and many of them are surrounded by a zone of congestion. On section they are seen to be composed of a firm pinkish gray tissue surrounding a central area of softening. There are a few subpleural petechiae on the lower lobes of the lungs (see Fig. 1).

The pericardial sack contains a small amount of clear yellowish fluid. The heart is of about normal size. The auricular epicardium is of a yellowish color. The aortic, mitral, and tricuspid valves appear normal. A yellowish fibrinous clot is loosely adherent to the walls of the right ventricle.

There is no fluid in the peritoneal cavity. The appendix is normal. The visceral peritoneum shows nothing abnormal. Intestines not opened. The spleen is considerably enlarged and of a fairly firm texture; its capsule is smooth and of a light purple color, mottled with areas of deep purple and these darker areas are seen on section to extend into its substance.

The right and left kidneys are alike, they are intensely congested, their capsules strip readily; each shows half a dozen subcapsular abscesses, 2-4 mm. in diameter; on section the cortex appears swollen and its markings are indistinct; section through some of the abscesses shows that some of them are surrounded by an area of intensely congested tissue. The adrenals are both enlarged and show marked congestion on section.

The liver is congested; just beneath the capsule in the center of the right lobe is an abscess about 3 mm. in diameter; the cut surface has a nutmeg appearance.

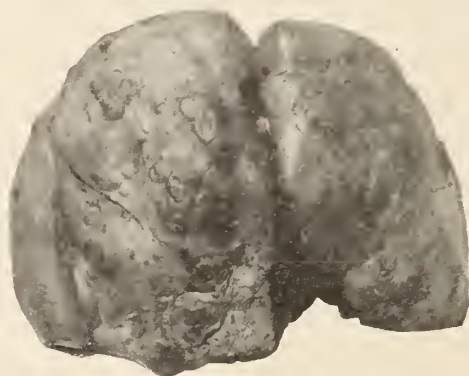


FIG. 1. Human lungs from fatal case of squirrel plague.

**Anatomic Diagnosis.**—Bubonic, lobular pneumonic, and pyemic plague; hydrothorax; passive congestion of the liver; acute nephritis, sub-acute splenitis; multiple abscesses in the regional glands, lungs, liver, and kidneys.

Smears made from one of the lung nodules showed a few typical bipolar bacilli and a few involution forms bearing a strong resemblance to those seen in the early growth of the plague bacillus on salt agar. We might state here that the only other occasions on which we have seen these involution forms from animal tissues have been in a few cases of squirrel plague. We have never seen them in human plague, rat plague, nor in the guinea-pig.

The bacteriological examination of one of the lung nodules taken at the post mortem examination yielded the following results:

A pure culture of *B. pestis* was isolated from one of the lung

nodules by culture; a guinea-pig inoculated by the cutaneous method with a piece of a lung nodule died on the fifth day with typical lesions of plague and a culture of the plague bacillus was isolated from its liver. Another guinea-pig was inoculated from the inguinal bubo. This pig was killed on the 14th day and found to be entirely normal.

Surgeon Rupert Blue has suggested to us that, in his experience, cases of plague in man, in which the infection was derived from squirrels, are apt to be less virulent than where the infection has been derived presumably from rats. The present case might, perhaps, lend some support to this view. We have seen but one other case of plague which was undoubtedly due to squirrel infection. In that case the patient recovered after a long and severe illness and after having developed a general plague septicemia. Two cases of plague occurred a year ago in Contra Costa County, California, both of which possibly derived their infection from squirrels, and these cases died of acute plague on the third and fourth day of the disease respectively. In these four cases, two of which certainly and two very likely derived their infection from squirrels, the bubo was situated in the axillary region in each case.

In the majority of cases of human plague due to rat infection the bubo is in the inguinal region.

We would call special attention to the following points:

1. This is the first case of undoubted squirrel plague in man which has come to autopsy in America.

2. The lesions were similar to those of sub acute plague in guinea-pigs, rats, and squirrels. We have never seen lesions of the same nature in any other case of human plague; in fact, without any knowledge of the previous history of the case one would scarcely have suspected plague infection at autopsy.

We wish to acknowledge our indebtedness to Surgeon Rupert Blue, Dr. W. A. Clark, superintendent of the Alameda County Infirmary, and Dr. H. E. Morrison, of Niles, for data used in preparing the present report.

## PATHOLOGY AND BACTERIOLOGY OF PLAGUE IN SQUIRRELS.\*

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THIS report is based upon 70 plague-infected ground squirrels (*Citellus beecheyi*) which have come under observation in California during the months of June, July, and August, 1909. During this period approximately 200 squirrels have been reported as plague infected to Surgeon Rupert Blue, Public Health and Marine Hospital Service, commanding the plague suppressive measures. In the case of the majority of these, the diagnosis was based upon the gross lesions alone or upon the gross lesions in addition to the result of the microscopical examination of the smear preparations from the buboes or from other tissues. In the case of 68 of the squirrels, however, tissues were inoculated into laboratory animals and when these animals died, cultures were made from their tissues and the specific bacillus isolated in about 30 cases. In the other cases the postmortem lesions presented by the laboratory animals were so characteristic of plague that it did not seem necessary to make cultures from them. In a few cases the cultures were hopelessly contaminated and the effort to isolate the plague bacillus was abandoned.

In two cases the bacillus was isolated directly from the tissues of a naturally infected ground squirrel. Indeed the attempt to recover the plague bacillus in this manner has been made on only about a half-dozen occasions. The reason that the effort was not made to isolate the bacillus directly from the naturally infected animals more frequently, was because of the fact that by the time we received the squirrels at the laboratory in San Francisco they were at least 24 hours old; sometimes 48 or 72 hours had elapsed after the animal had been shot. As a result of this delay the tissues were usually badly contaminated with saprophytic organisms.

It was our intention in the early part of the work to verify by bacteriological methods at least one squirrel from each ranch on

\* Received for publication October 6, 1909.

which infected animals were found, and this program has been faithfully carried out in nearly every case. However, as the number of infected animals received increased, in a few instances it was found impracticable to do this on account of lack of sufficient cages and of space in which to keep inoculated rats and guinea-pigs.

**Mode of Handling and Examining.**—A few of the squirrels were found dead by the men engaged in the anti-squirrel operations in Contra Costa and adjacent counties, a few were caught in traps, but the great majority were shot. These squirrels were packed in heavy sheet tin cans, each squirrel having been tagged to show the date on which it was taken and the person by whom it was shot, trapped, or found. The cans containing the squirrels were shipped to the Federal Plague Laboratory at San Francisco, a little chloroform having been placed in each can so that any ectoparasites might be killed. When the cans reached the laboratory a one per cent solution of trikresol or some similar disinfectant was used in sufficient quantity to cover the squirrels, after which they were nailed to shingles, the address recorded, and the animals dissected.

During the first few weeks of the work the medical officer personally examined every squirrel that was dissected. Later, as the number increased, it was found impracticable to do this and only those squirrels were examined that were laid aside by the laboratory assistants as suspicious. It is believed that no very material error resulted from this procedure, as during the whole of the time that all of the squirrels were scrutinized by the physician but two infected ones were found that had passed the men who dissected them. As soon as a suspicious lesion was found by an attendant, the dissection was suspended and the animal was laid aside for further and more detailed examination.

The location of lymphatic glands of ground squirrels is a point worth considering. The cervical, axillary, and pelvic glands are easily distinguished, being found in the usual situation of these glands in other rodents (rats and guinea-pigs). In the case of the inguinal glands, however, the location is somewhat different. In the squirrel there is a lymph node lying immediately above the pubis on each side and very near the middle line. In the ordinary dissection, this gland is very apt to be overlooked and as it is one which is very frequently involved in plague, it is important that it be exposed. We first learned of the location of this structure in doing certain work in the artificial infection of squirrels with plague. In addition to this gland, which we have for the sake of convenience called "the median inguinal gland," there is a chain of glands lying rather far back in the groin, sometimes extending nearly to the vertebral column. These structures we have called "the posterior inguinal glands." The great majority of squirrels do not have glands in the same

location in the inguinal region as do rats and guinea-pigs; that is to say, about the middle of the groin.

#### THE GROSS LESIONS OF NATURAL SQUIRREL PLAGUE.

The lesions of four plague-infected squirrels were described by Wherry,<sup>1</sup> who also reviews the literature on squirrel plague.

**Buboes.**—The external appearances of buboes: It is not an unusual occurrence to be able to make a probable diagnosis of squirrel plague before an incision is made into the body. A swelling in the region of one of the superficial glands often gives a clue to the condition that will be found.

The buboes found in squirrels may be divided into two classes, first, those in which the gland is distinctly caseous; and second, those in which the gland is more or less purulent. In our experience thus far the latter has been much more frequently met than the former.

In the first class of cases, that is, in those with caseous buboes, the gland is rather firm and is usually yellowish red in color. There is frequently a small amount of hemorrhage into the surrounding tissue, and the contents may readily be shelled out when the gland is split across. These glands are usually about 1 c.c. in length by 0.5 c.c. in width. Smears made from buboes of this nature almost invariably show a large number of bacilli which agree in size, shape, and staining reactions with the plague bacillus. Very frequently numerous "coccoid" forms are present. The observation may be merely a coincidence but this class of buboes was found rather more frequently in the early part of our work than in the latter part; in fact now such buboes are rather rare, while during the first two weeks in which plague infected squirrels were found they were fairly common.

The second class of buboes (purulent) are quite different from the preceding. The lymph gland may be enlarged and succulent throughout, with the exception of one or more purulent foci no larger than a mustard seed. In other cases the purulent foci are very much larger, sometimes as large as a pea, and the gland may be entirely honeycombed with such small abscesses. In still other cases, the gland or chain of glands will be converted into a large pus sack, sometimes considerably more than 1 c.c. in diameter. In these purulent buboes

<sup>1</sup> *Jour. Infect. Dis.*, 1908, 5, pp. 485-533.



pest-like bacilli either are very scarce, or as is true in the majority of cases, none at all are to be found by microscopical examination.

In two or three cases, we have seen in smears from the bubo as well as in smears from the caseous lesions in the internal organs, objects which very strongly resemble the "involution" forms of plague bacilli when the organism is grown on salt agar. On one occasion we have seen these forms from a lung nodule of a case of human plague which died on the 16th day of the disease. This case was infected from squirrels.

In a considerable number of cases, one is in doubt as to whether a given bubo should be classed as caseous or as purulent. The content is a yellow, very tenacious, or flaky mass, which probably represents a transition stage between the purely caseous and the frankly purulent gland.

**Spleen Lesions.**—Enlargement of the spleen alone we have learned to regard as of but little significance because the size of the organ in the ground squirrel varies very markedly in the apparently normal animals. Indeed the largest spleens we have seen were in animals which were not suspected of being plague infected and which by inoculation experiments have proven to be free from the disease. However, in the plague infected animals the organ is frequently enlarged. It is usually deep red in color in contrast to the lilac tinge seen in the normal squirrel. Occasionally it is slate colored. The most common lesions of plague are caseous or purulent foci varying in size from a mustard seed to a mass as large as the last joint of one's index finger, and in number from one to a hundred or even more. In a few cases the caseous nodules seem to be in the process of organization.

Less frequently hemorrhagic areas are to be found which vary in size as do the caseous foci.

**Liver Lesions.**—In the liver are found purulent and caseous foci similar in size and number to those in the spleen. In a few cases the liver tissue has been very largely replaced by purulent masses, and in some of these cases it has been noted that there were large purulent masses scattered through the omentum and the mesentery. The organ is often adherent to the diaphragm.

**Lung Lesions.**—The lung lesions may be either caseous or puru-

lent, and vary in size from a pin's head to a pea. Occasionally much larger nodules are found. The lesions are frequently surrounded by a deep red zone of congestion, and upon section are either caseous or purulent. Pleural adhesions over the site of the nodules are common.

**Kidney Lesions.**—Pea-sized abscesses have been found in a few cases.

**General Appearance of the Squirrel.**—Those animals in which lesions are very extensive are often markedly emaciated; sometimes in these cases the tissues are found to be jaundiced. On the other hand, in most of the cases in which the macroscopical evidence of plague is limited to a purulent gland, the animals are quite fat.

#### REMARKS ON THE GENERAL NATURE OF THE LESIONS.

The lesions of plague in most animals are generally uniform and well defined. The variation between individual cases in man, in the guinea-pig, and in the rat is slight and unimportant. These animals usually die between the third and the sixth or seventh day of the disease.

In the squirrel, however, there is a most marked diversity in the lesions found in different cases. They range all the way from a very small innocent-looking purulent lymphatic gland, up to extensive necrotic processes involving several of the chains of glands and almost all of the internal organs. Two examples illustrating the extremes of lesions found are given here.

The first case is one in which the lesions were very slight.

**Squirrel No. 68.**—Half grown female; shot on July 17, 1909, by C. The lesions are recorded as follows:

*Lymphatic glands:* Right median inguinal gland enlarged and shows several very small yellowish purulent points. The other glands are normal. Liver, spleen, and lungs negative.

*Smears:* Entirely negative for *B. pestis*. A guinea-pig (No. 68A) inoculated from the bubo material died on the third day with entirely characteristic lesions of plague and a pure culture of *B. pestis* was isolated from its tissues.

The next is an example of a squirrel showing very marked and widespread pathological lesions.

**Squirrel No. 7.**—Grown female, found dead by W. F. R. on June 18, 1909.

*Lymphatic glands:* Right cervical and bilateral median inguinal buboes each a little smaller than a pea and each full of yellow, creamy pus.

*Liver:* Numerous abscesses up to 2 mm. in diameter.

*Spleen:* Several distinct abscesses, largest about 1 cm. in diameter, numerous minute purulent points.

*Lungs:* About a dozen caseous foci, each one approximately 1 mm. in diameter.

*Smears* from the inguinal bubo show a few suspicious-looking organisms; from the liver show very typical pest-like bacilli.

On the surface of the spleen and of the liver there is found a considerable quantity of yellowish fibrinous exudate.

A guinea-pig inoculated subcutaneously from the liver of this squirrel died on the fourth day with characteristic lesions of plague; and a white rat inoculated by the cutaneous method also died on the fourth day, with the usual lesions of plague in the rat.

The question of course arises as to whether, especially in the cases where the anatomical changes are very extensive and widespread, some other organism plays a part in the production of the lesions. We can only say that these lesions have been invariably associated with pest bacilli. When the tissues reached us they were in such condition as to preclude a satisfactory bacteriological examination for other pathogenic organisms.

#### FREQUENCY OF VARIOUS LESIONS.

The gross lesions in these 70 cases of squirrel plague may be conveniently tabulated as follows:

Total showing buboes.....	58	(83%)
	{	
	Cervical.....	17
	Median, inguinal.....	14
Buboes, single in ....	Axillary.....	8
Buboes, multiple in..	Pelvic.....	5
Without buboes.....	12	(17%)
Total.....	70	

In the 44 cases with single buboes lesions of the internal organs were present as follows:

Spleen.....	10 times
Liver.....	6 "
Lungs.....	5 "
Spleen, liver, and lungs.....	3 "

Thirty-two (32) of the 44 cases had no lesions whatever beyond the bubo.

Multiple buboes (14) as follows:

Bilateral inguinal buboes, 7	{	Bilateral median inguinal only.....	2
		Bilateral median inguinal and bilateral pelvic.....	2
		Bilateral median inguinal and right cervical.....	1
		Bilateral median inguinal and right post inguinal...	1
		Bilateral median inguinal right post inguinal and right axillary.....	1

Bilateral submaxillary, 1

Median inguinal buboes and others, 4	{ Median inguinal and pelvic .....	3
	{ Median inguinal and post inguinal.....	1

Median inguinal and left axillary, 1

Median inguinal and right post inguinal and right submaxillary, 1

In these 14 cases, lesions of the internal organs were present as follows:

Spleen.....	3
Liver.....	5
Lungs.....	5
Spleen, liver, and lungs.....	2

Of these cases eight had no lesions whatever beyond the buboes. It will be observed that a much larger proportion of the cases with multiple buboes had lesions of the internal organs than of those with single buboes.

Cases without buboes (12) had lesions as follows:

Spleen.....	12
Liver.....	9
Lungs.....	2
Spleen, liver, and lungs.....	1

The anatomical findings would seem to justify the following observations:

It is probably fair to assume that all cases having lesions of the internal organs have been at some time septicemic.

In the cases where the only lesion is a bubo, it is very unlikely that the bacilli have ever invaded the blood stream.

**Microscopical Examination of Smears.**—Smears from one or more tissues were made in each case. The smears were stained with methylene blue or thionin, and a search made for pest-like bacilli. The results of the examinations are conveniently tabulated as follows:

Positive .....	17
Suspicious and doubtful.....	4
Negative.....	47
Not recorded .....	2
Total.....	70

On several occasions the smear from a bubo was negative while the organism was found in the splenic or hepatic nodules.

A word of explanation is necessary in regard to the recording of the results of the examination of stained smear preparations. In my experience with plague in man and in rodents, it is not advisable to spend much time looking for a few isolated organisms having the morphology of the pest bacillus, nor is any great stress laid upon the presence of a very few even quite characteristic organisms. Judging

by these standards, in the great majority of cases the smears were regarded and recorded as negative. In a smaller percentage of the cases the examination of smears revealed such a large number of very typical pest-like bacilli as to make it almost certain that they were actually *B. pestis*. None of the cases included in this report depended on the examination of smears alone to support the diagnosis made from the gross anatomical appearances of the squirrel.

*Results of animal inoculations.*—As I have said in the introductory paragraph, the diagnosis in 68 of the 70 cases has been made (or confirmed) by animal inoculation. It is not my purpose to discuss here the lesions of plague in laboratory animals, but I will state that the lesions produced were those characteristic of plague in rats and in guinea-pigs.

In a considerable number of cases the inoculated animals died of subacute plague due probably to low virulence (or small number) of the organisms present in the squirrel tissues. In a great many cases, however, as will be seen from the tables below, the laboratory animals died of acute plague in the usual time, three, four, or five days.

#### CULTURAL CHARACTERISTICS OF THE ORGANISM ISOLATED.

The routine method of dealing with cultures was as follows:

Cultures were made upon agar slants directly from the tissues of the squirrel or infected laboratory animal. These were examined at the end of 48 hours. If the growth was profuse or if the organism was evidently a chromogen, the culture was discarded without any further investigation. If the growth was found to be translucent, shiny, and if it adhered to the needle when touched, it was regarded as of sufficient importance to make subcultures on other media. Subcultures were always made on broth, and on salt agar; at the end of 48 hours a flocculent precipitate was observed in the broth culture and after a few days a delicate surface film which settled when the tube was agitated. Subcultures are also made on 3 per cent salt agar. A smear from this culture was examined microscopically after from 18 to 24 hours, when if the culture be one of *B. pestis* characteristic involution forms will be found. In the majority of cases subcultures were also made in litmus milk, which was usually rendered a trifle less alkaline at the end of 48 hours. The litmus milk cultures were also kept for at least 10 days for the purpose excluding *B. pseudo-tuberculosis rodentium* (Pfeiffer) which renders the milk very alkaline. In many cases we have also planted the organism in Dunham's solution, but have never observed the formation of indol. The plague bacillus, as is well known, does not produce indol. A few cultures have also been made on gelatin for the purpose of ascertaining the reaction to this medium.

Such a simple technic as that described is not to be recommended in any special case in which it is vitally important to isolate the

bacillus from the tissue. In such cases it is better to use the plate method, as it is obvious that a small contamination may cause the loss of a culture in the method I describe. This was not a matter of special concern in the present work, as the bacillus had been isolated in a sufficient number of cases to leave no doubt as to the specific nature of the organism we were dealing with. The growth of plague bacillus on agar is so characteristic that a judgment based upon the appearance and nature of the growth on that medium very exceptionally proves to be erroneous.

We have used Yersin's anti-pest-serum for the purpose of verifying the diagnosis in this series of cases in but one instance. In this case, which was that of the first squirrel found infected during the present year (1909), two guinea-pigs were vaccinated from the bubo of the squirrel. One guinea-pig which weighed 290 gm. had been injected previously intraperitoneally with 3 c.c. of an old specimen of anti-plague serum. This guinea-pig died on the eleventh day. The internal organs were free from any lesions, but the superficial glands were all enormously enlarged and full of caseous material. A pure culture of the plague bacillus was isolated from one of these glands. In other words, this guinea-pig died of chronic or subacute plague. The other guinea-pig (the control), which weighed 335 gm., died on the fifth day with the typical lesions of acute plague and a pure culture of the bacillus was isolated from the liver. This one example is not insisted upon as being convincing in showing the protective power of anti-plague serum against the organism found in this particular squirrel, but it is very suggestive.

In the first case of plague found among the ground squirrels in California (1908) the subject of the relation of the infecting organism to anti-pest serum was worked out rather more carefully with results that I think would convince anyone that the serum exercised a high protective power. The results have been reported in the *Public Health Reports*.<sup>1</sup>

#### COMPARATIVE VIRULENCE FOR GUINEA-PIGS AND WHITE RATS.

In some of the early cases it was found that a white rat inoculated from a squirrel survived, while a guinea-pig inoculated from the

<sup>1</sup> Vol. 23, No. 37, September 11, 1909, p. 1292.



same tissue succumbed to plague. With a view of determining exactly the comparative virulence for white rats and guinea-pigs, two series of rodents were inoculated. The results are shown in the following table:

TABLE 1.

No. of Squirrel	Guinea-Pig Day of Death	White Rat—Day of Death
Inoculated Cutaneously		
1 (bubo)†.....	5 (c.)*	3 (c.)
65 (bubo).....	8	Killed on 12th day; subacute plague
66 (bubo).....	6 (c.)	Killed on 12th day; subacute plague
67 (liver nodule).....	11 (c.)	Killed on 12th day; no lesions
68 (bubo).....	4	3 (c.)
73 (bubo).....	4 (c.)	4 (c.)
Inoculated Subcutaneously		
1 (bubo).....	4 (c.)	2
69 (bubo).....	5 (c.)	4
70 (bubo).....	8	Killed on 11th day; no lesions
71 (bubo).....	4 (c.)	5
72 (bubo).....	5	3 (c.)

\* (c.) indicates that a culture of *B. pestis* was isolated from the inoculated animal.

† The word in parenthesis after the number of the squirrel, shows the squirrel tissues used for the inoculations. These explanations also apply to the table on p. 686.

In each case the same piece of tissue was used to vaccinate or inoculate a guinea-pig, and to vaccinate or inoculate the corresponding white rat. It will be seen in both the subcutaneous and the cutaneous series that the infection was decidedly more virulent for guinea-pigs than for white rats as all of the guinea-pigs died of plague, while two of the white rats had no lesions whatever, and two developed subacute plague.

#### VIRULENCE FOR GUINEA-PIGS BY DIFFERENT MODES OF INOCULATION.

In order to determine the value of the cutaneous (vaccination) method of inoculation in squirrel plague two series of guinea-pigs were inoculated.

The technic was as follows: A piece of the suspected tissue was vigorously rubbed into the shaven belly of a guinea-pig. The same piece of tissue was then thrust into a pocket beneath the skin of another guinea-pig.

The table shows that of the 12 cases, two would have been missed by depending solely upon the cutaneous method of inoculation. Guinea-pigs of approximately the same weight were used in each case.

The question has been raised as to whether the plague encountered among these ground squirrels is identical in every respect with the plague found in rats and human beings. In answer to this question we can only say that either directly or by the use of laboratory animals we have isolated from these squirrels an organism which answers all of the cultural and pathogenic requirements for *B. pestis*.

The only difference observed between the strains of plague isolated from these squirrels and that from rats and man is, that

TABLE 2.  
VIRULENCE FOR GUINEA-PIGS BY CUTANEOUS AND SUBCUTANEOUS INOCULATION.

No. of Squirrel	Guinea-Pig Inoculated Cutaneously Day of Death	Guinea-Pig Inoculated Subcutaneously Day of Death
1 (bubo)	5	4
6 (bubo)	5 (c.)*	5
43 (bubo)	4 (c.)	3
59 (bubo)	7	6
77 (bubo)	6	4
111 (bubo)	5 (c.)	6
120 (spleen)	Alive and well 12th day	6
123 (bubo)	6	6 (c.)
127 (bubo)	6	4 (c.)
135 (bubo)	Alive and well 16th day	5
137 (spleen)	5 (c.)	5
140 (spleen)	5 (c.)	4

the squirrel strain appears to be somewhat less virulent than the others. This reduction of virulence is shown by the fact that a considerable series of inoculated guinea-pigs lived on an average somewhat longer than guinea-pigs inoculated from natural plague rats or from cases of human plague; and second, the fact that a considerable percentage of the white rats are immune wholly or in part to this strain of the plague bacillus. In my experience I have not encountered among white rats a case of immunity to rat plague or to human plague, though such cases are said to occur. Cross immunization experiments are now in progress to determine the interrelationship of human plague, rat plague, and squirrel plague.

#### SUMMARY AND CONCLUSIONS.

Plague in the ground squirrel is a disease that is readily recognized by the gross anatomical changes it produces.

The commonest lesion, and often the only one, is a bubo.

Many of the cases are probably examples of subacute or chronic plague.

In many cases the bacilli found in squirrel plague are highly virulent for guinea-pigs and white rats, in other cases the virulence is somewhat reduced.

Smear preparations are negative for pest-like bacilli in the majority of cases.

It is unsafe to trust to the cutaneous method of inoculation alone, as it will sometimes fail when the subcutaneous method yields positive results.

## ON THE RESISTANCE OF HUMAN ERYTHROCYTES TO COBRA VENOM.\*

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It was pointed out by the author<sup>1</sup> in a previous paper that the varying resistance of red cells to hemolytic agents is very probably to be regarded as an adaptive or protective mechanism. In the attempt to verify this hypothesis, a number of hemolytic agents have been tested against a series of human red cells derived from individuals suffering with different types of disease. Of these hemolytic agents, cobra venom<sup>2</sup> gave results which seem to be of especial interest. Similar results, but much less striking in character, were obtained with saponin and digitonin. Rattlesnake venom has not been sufficiently tested to permit of a full report. In this connection, it was observed by H. Sachs<sup>3</sup> that red cells taken from different individuals of the same species manifest a varying degree of resistance to the action of cobra venom, and he suggested that the venom might be used as an indicator of the amount of lecithin present in various tissues. Bang has recently corroborated this observation as to individual variations in resistance to cobra venom.

**Method.**—Blood is drawn with an aspirating syringe from one of the veins at the bend of the elbow, in amounts of not less than 10 c.c. Of this blood at least 2 c.c. is expelled into a test tube containing about the same quantity of a two per cent solution of sodium citrate; the remainder is kept and the serum tested according to the method of Wasserman or Noguchi. The corpuscles should not be prepared by shaking. The citrated plasma may be prepared for use on the same day, or may be kept as long as 48 hours in the ice box. It is centrifuged four times, the corpuscles being washed in a 0.9 per cent solution of common salt. In carrying out this process of washing, it is essential to remove the serum very thoroughly, inasmuch as it interferes very decidedly, even in slight traces, with the action of cobra venom. The washed cells are made up into a four per cent suspension in 0.9 per cent common salt solution. The cells are centrifuged in very accurately graduated centrifuge tubes, and their accurate dilution is a matter of very great importance. The cell suspensions may be tested when fresh, or after a brief sojourn in the ice chest. If they are to be tested

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<sup>1</sup> *Proc. Soc. Exper. Biol. and Med.*, 1909, 6, pp. 49-51.

<sup>2</sup> I am indebted to Drs. Flexner and Noguchi for samples of venom.

<sup>3</sup> Kraus and Levaditi, *Handbuch*, Jena, 1907, 1, pp. 254, 258.

immediately, the salt solution in which they are to be suspended should be kept in the ice chest until used; in this manner, the initial temperatures at which the mixtures of cobra venom and cells are made is always approximately the same. The cobra venom, which is kept in the dried condition, is made up very accurately in small quantities into a 0.05 per cent solution in 0.9 per cent solution of common salt. From this stock solution, higher dilutions are prepared as required, and are kept at a constant low temperature. The solutions of venom kept in this manner remain of a constant strength for several days at least. The resistance of the corpuscles to cobra venom may be tested roughly within an hour, or more delicately after 24 hours. If the rapid results are desired, venom solutions of 1 in 8,000, and of 1 in 15,000 are prepared. If the results are to be read on the following day, four solutions are prepared, namely, 1 in 10,000, 20,000, 30,000, and 40,000. One c.c. of the red cells is added to 1 c.c. of the venom solutions. If the rapid method is employed, the mixtures are incubated at 40° for 30 minutes, and all cells which show no hemolysis in 1 to 8,000 after this time are considered positive, while all which show destruction at 1 to 15,000 are considered negative. If the second method is employed, and this is the more delicate of the two, the mixtures are incubated for one hour, after which a preliminary inspection will almost invariably reveal the final results of the tests. At this stage, cells showing no hemolysis with 1 to 10,000 venom are strongly positive, cells showing no hemolysis with 1 to 20,000 are positive, cells showing very slight hemolysis at 1 to 30,000 are weakly positive; cells showing moderate destruction at 1 to 30,000 are negative, and cells showing the least appreciable hemolysis at 1 to 40,000 are strongly negative. After a preliminary reading, the mixtures are again shaken, and are kept in the ice box over night, the final readings being made on the following morning. At the final reading, any cells which do not show complete hemolysis at 1 to 1,000 are regarded as strongly positive; cells only moderately hemolyzed at 1 to 20,000 are regarded as positive; cells showing complete destruction at 1 to 30,000 are regarded as negative; cells showing complete destruction at 1 to 40,000 are regarded as extremely susceptible to the venom and are denoted in the abbreviated descriptions as "— — — —". The meaning of the various degrees of hemolysis can easily be deduced from these data. The results should be absolutely concordant for the whole series of dilutions; if there appear to be discrepancies, the entire set of tubes should be rejected and the mixtures made afresh. The figures given above hold, of course, for the venom which formed the basis of these experiments; it is probable that other samples might differ in strength. At all events, a preliminary titration against corpuscles derived from cases of known lues and controls suffices to determine the values for any given sample. Inasmuch as the strength of the dried venom does not vary, this preliminary titration gives a constant standard, which is practically permanent, since one gram of venom suffices for 5,000 complete tests. It should furthermore be stated that the standardization is different for infants as compared with adults. It was long ago pointed out by Sachs that the resistance to various hemolytic agents, including venom, increases materially in the change from early to adult life in some of the lower species. The same condition holds true of man. In case of infants and young children, it is found that normal red cells hemolyze much more readily than those of adults. The cells, however, acquire the same degree of resistance as do those of adults in disease.

The cases which form the basis of the present communication number 191. They were obtained from the wards of the German

Hospital, from the neurological dispensary of the Presbyterian Hospital, through the kindness of Dr. Schlapp, and from the genito-urinary and other departments of the Cornell University dispensary, through the kindness of Dr. Hastings and his assistants. The material was uniformly submitted to the procedures described above. The degree of hemolysis in the various tubes was described, according to the notation in common use, as Complete (C), incomplete (+++), very marked (++) , moderate (+), slight (sl), and absent (-). The interpretation of these degrees of hemolysis for the different concentrations of venom has been previously described. In interpreting the degree of resistance of the red cells, three independent criteria are employed, which, however, practically always vary in the same direction and to a similar extent, so that the practical gauging of the results is very much simpler and more accurate than in most hemolytic experiments. These criteria are: the rapidity with which hemolysis begins, the degree of completeness at the end of the period of observation, and the tendency of the red cells, as the test proceeds, either to sink rapidly to the bottom of the tubes or to remain suspended in the fluid. In cases in which the red cells are susceptible, the hemolysis begins early, often within the first half-hour in the incubator; it tends to be marked in the moderate dilutions of venom at the end of the experiment; and the undestroyed red cells sink rapidly to the bottom of the tubes. These conditions are reversed in the case of resistant corpuscles. The cause of the change in the relative specific gravities of the red cells and the fluid has not been determined; it is certainly a very marked feature.

In studying the blood from a large number of human individuals, some of them normal, others afflicted with a variety of acute or chronic diseases, it is at once evident that there is a certain variability in the resistance of the red cells to the action of cobra venom. That this should be the case is not entirely surprising. It might *a priori* be expected that there would be some variability among tissue cells in this respect, as there is in all others. Indeed, it has been pointed out by a number of observers that such variability toward the action of other hemolysins than cobra venom exists among the individuals not only of the human but of other species. In one respect, however, namely, the very wide margin of difference between susceptible and



resistant cells, variability toward cobra venom differs from that toward most other hemolysins. The condition which presents the most marked degree of resistance to the action of cobra venom is syphilis, after it has passed the primary stage. Normal individuals, and various diseased conditions manifest variability of resistance within certain narrow limits, but this never approaches the degree of resistance which is characteristic of syphilis. It is this fact, which makes it possible to construct a cobra spectrum, so to speak, with all the cases of syphilis grouped at one end and those of other conditions grouped in a graded series below it. On this account, also, it has been possible to group all cells which manifest resistance of a certain grade as positive, and all others which are distinctly less resistant as negative. Upon analysis it is found that all of the cells of the "positive" series are derived from advanced syphilitics, while all those of the "negative" series belong to other conditions.

In analyzing the results of the present series of tests, the diagnosis of cases has been made chiefly from the clinical history and findings. In all doubtful cases, the serum of the cases has also been tested according to the method of Wasserman and Noguchi.<sup>1</sup> The results may be considered from various standpoints. Taking, first, the cases with a distinct history of syphilis, and marked evidences of an active syphilitic process, these are found to be uniformly marked by resistance to the cobra venom, and to fall into the group of positives. Of this type of cases, the series includes patients with syphilis of the skin, of the eye, of the central nervous system, of the aorta and great vessels, and of the bones and joints. The infection has ranged from several months to 21 years previously. In very few of these cases has a negative reaction been obtained. In almost all of them the Noguchi or Wasserman reactions were positive.

Then there is a considerable number of cases which have given a history of syphilitic infection, generally from five to forty years previously, who presented at the time of examination no evidences of an active syphilitic process. Some of these cases had been treated, and some had not. Of this group, the majority of the treated cases gave a negative reaction. Of the untreated cases, a considerable

<sup>1</sup> For help in this regard, I am indebted to Dr. Kaplan of the Montefiore Home Laboratory, and to Drs. Warren and Schwarz, of the Cornell Clinical Laboratory. Recently Dr. Noguchi has examined sera for me.

number gave a positive reaction, which varied from a moderate to a marked degree. The results of the Wasserman and of the Noguchi reaction in this group of cases were not in complete accordance with the resistance reactions. In very few of the old, inactive, untreated cases, did they give a positive result. There is another group of cases in which there is no history of syphilitic infection, but in which, on clinical grounds, it seems necessary to infer a syphilitic origin of the disease. Of such cases, the present series includes such different conditions as habitual abortion, numerous stillbirths, hereditary infantile disease, certain bone diseases, certain types of cirrhosis of the liver, and certain nervous diseases such as tumor of the brain. This group excludes cases of *tabes dorsalis*. Of these cases, the majority were positive according to the cobra venom test, thus confirming the clinical diagnosis; they reacted, as a rule, in the same manner to the Wasserman method. In a very small number of cases, the Wasserman reaction was positive, while the venom test was negative; it is not yet possible to report on the true status of these cases.

In the cases taken as controls, it was twice possible to obtain a positive reaction. One of these individuals, who was suffering from a large cancer of the stomach, subsequently confessed to a syphilitic infection 20 years previously, which had again broken out 12 years later. The other, a case of Stokes-Adam's disease in the German Hospital, persistently denied syphilitic infection; in this case, the Wasserman and Noguchi tests were positive; the results of the therapeutic tests are not yet conclusive. Of the cases in which it was not possible to obtain a syphilitic history, but in which the clinical condition suggested syphilis, a very considerable proportion was constituted by the cases of locomotor ataxia. These gave a very high percentage of positive results with Wasserman reaction or the Noguchi modification, whereas a smaller number indicated a luetic origin with the cobra venom test.

The very early cases of lues, with chancre, or the first eruption, are often distinguished by the opposite condition. In them the cells are more than normally susceptible to the action of the venom. Frequently, at the end of one hour incubation, they show advanced hemolysis in the tubes containing 1 to 30,000 venom, and by the next

day often all four tubes are completely hemolyzed. Within one to six months, however, from the appearance of the primary sore, the condition of increased resistance of the cells is manifest, and is apparently retained for years, unless abolished by treatment. It has not been possible to follow the development of this change of resistance in any individual case, owing to the obvious fact that cases are treated as soon as diagnosed. The above deductions are drawn from the observation of a series of early cases at various stages. The effect of treatment by means of mercury is gradually to abolish the resistance of the red cells. This effect, however, is only slowly accomplished. This evidence of syphilis, indeed, persists sometimes for weeks, or even months, after treatment has abolished the Wasserman reaction. The bearing of this fact on therapeutic procedures will be subsequently considered.

The non-syphilitic cases of this series have not, with the few questionable exceptions noted above, given any positive reactions. There have occasionally in this class been cases which verged on the positive reaction but none which could be unquestionably grouped as such. Among these have been cardio-nephritic, cancerous, and tuberculous cases; they have not as yet caused any actual confusion in the determinations. It should be stated, however, that the series is still too small to permit of a wide generalization excluding all non-syphilitic cases. For example, no typhoids or pneumonias had been tested.

There is a certain class of cases which has been found to give a positive Wasserman reaction, in spite of the fact that they are well known not to be syphilitic. These are, for example, cases of scarlet fever, leprosy, scleroderma, and some cases of polycythemia. Of scarlet fever six cases, of scleroderma one, and of polycythemia two, have been examined, and none of them has evinced an increased resistance of the red cells to cobra venom. In fact, the scarlet fever cases rather showed supersusceptibility to the venom.

Such is, in brief, a summary of the findings in the reactions done up to the present time. The results may be considered from either a practical or a purely theoretical standpoint. Practically, it would seem superfluous to provide another blood test for syphilis, when the Wasserman and the Noguchi reactions seem so completely to fulfil

the needs of the clinic. On the other hand, in certain particulars, cobra venom might offer certain advantages over the Wasserman reaction, should it prove as reliable a criterion in a larger collection of cases as it has in the present series. These advantages appear to be the following: The method is much simpler and the labor much less. Less blood is required, so that a few drops from the lobe of the ear suffice for a reaction; this is of some importance in infants. Cases of scarlatina and of leprosy do not offer a source of confusion. The reaction is more marked in old, apparently dormant cases. The reaction persists much longer after mercurialization, thus offering a further diagnostic and therapeutic test. The reaction is possible in cases of jaundice, whereas the Wasserman is not. Aside from its practical application, the reaction suggests certain problems of a purely theoretical nature. It is apparently the first case of a constant and characteristic alteration in tissue cells, as opposed to the serum, in response to a given pathological condition. The question is whether this alteration is to be regarded as a direct result of the action of the syphilitic virus, or as a reactive and protective response of the organism to the action of that virus. The former alternative suggests a comparison with the effects of the hypodermic injection of phenyl-hydrazin, which produces a rapid and striking increase in the resistance of the red blood cells to all hemolytic agents. On the other hand, there are certain features of the present case which seem to oppose such an interpretation. The preliminary period of hypersusceptibility would seem to indicate that the resistance cannot possibly be the result of the immediate action of the syphilitic virus on the red cells, but that it is a reactive phenomenon. In this connection, the author may recall the fact that about a year ago he published a somewhat similar observation drawn from experimental data. It was found that repeated hypodermic injections of saponin over a long period of time in rabbits results in the production of red blood cells which possess a heightened degree of resistance to saponin, but to no other hemolytic agents; this period of increased resistance is, however, preceded by a short but distinct period of hypersusceptibility to the poison. It was suggested at the time that the hypersusceptible cells represented the first generations of red cells which had come into contact with the poison; that they had been partially

charged with it, or partially injured by it, and were therefore more easily destroyed. The later and resistant generations were believed to represent a reactive process. It is not difficult to conceive of some such process in the case of saponin, but it is certainly a very different matter to explain the action of syphilis in producing an increased resistance to so different a poison as cobra venom. It has been shown by repeated experiments that this resistance to cobra venom is specific, and that the syphilitic red cells show no increase of resistance to other hemolytic agents. It is perhaps permissible to suggest that the luetic virus attacks the same cellular constituent as does cobra venom. In the discussion of this possibility, one is forced unfortunately to rely on a considerable number of only partially tested data, and the deductions are likely to be correspondingly inaccurate and unreliable. It has been found by Kyes and Sachs that cobra venom unites with lecithin to form a toxic cobra-lecithid. They also showed that this lecithin might be derived from the red blood cells themselves, where it was present as an "endocomplement." In conformity with this view, it would be possible to conceive that the amount of lecithin in the red cells had been diminished in luetic conditions, thus leading to the production of a smaller amount of the toxic cobra-lecithid in the red cells. That lues does in fact attack the lipoids of the body, and that the amount of lecithin which can be extracted from the tissues is less in syphilitic conditions than in normal individuals, is apparently well attested. On the other hand, it is possible, as has been personally suggested to me by Noguchi, that the sodium citrate plays an essential rôle in the reaction, as it has been shown to do in cobra hemolysis. This possibility is supported by the fact that shaken cells fail to exhibit the differences above described. Which, if any, of these possibilities represents the correct interpretation of the phenomena, can be determined only by further study of the conditions.





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